The Role of the Intestine in the Metabolism of Thyroid Hormones and in the Regulation of Thyroidal Status in the Rainbow Trout, *Oncorhynchus mykiss*

by

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THE UNIVERSITY OF MANITOBA

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Of

DOCTOR OF PHILOSOPHY

Table of Contents

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Abstract

In rainbow trout, peripheral tissues regulate thyroidal status, or the availability of 3',3,5-triiodothyronine (T_3). Hence, the intestine with access to thyroid hormones (TH) through diet, blood supply, biliary supply, and potential TH metabolism may contribute to thyroidal status.

To evaluate the role of the intestine in TH metabolism, assay conditions for *in vitro* TH metabolism in pyloric caeca (PC), middle intestine (MI), and distal intestine (DI) were characterized. In the intestine, mainly DI, thyroxine (T_4) complete outer-ring deiodination (T_4cORD), T_4ORD, T_4 inner-ring deiodination (T_4IRD), T_3ORD, T_3IRD, and iodoprotein (IP) production were active. Cofactor was not required.

To evaluate the role of the intestine in regulating TH metabolism during physiological challenges to thyroidal status, fish were fed T_4 or T_3 for 7 days. In MI, T_3 challenge increased T_4cORD, T_4IP, T_4IRD, and T_3ORD. In DI, T_4 challenge increased T_4ORD. T_3 challenge increased T_4ORD and T_3IRD. However, T4cORD and T_4IRD were always active. In both intestinal regions, T_3 was degraded and formation of T_3 was prevented.

To evaluate the role of the intestine in supplying TH, *in vivo* uptake of T_4 and T_3 from intestinal sections into enterohepatic tissues (liver, blood and gall bladder) was measured. If TH were accumulated in bile, enterohepatic circulation (EHC), or TH cycling from intestine to liver to blood and back to intestine, could occur. From the MI section, more T_3 than T_4 was absorbed. It did not contribute to T_4 or T_3 EHC. Acute increases of T_4 or T_3 were metabolized peripherally and cleared via bile. In MI-DI

section, more T_4 than T_3 was absorbed. T_4 EHC was greater than T_3 EHC. Acute increases in T_4 or T_3 increased T_4 or T_3 EHC.

To evaluate the role of the intestine in supplying TH after physiological challenges, alterations in *in vivo* uptake of T_4 and T_3 from MI-DI section into enterohepatic tissues were measured. T_4 challenge increased T_4 EHC, and T_3 and T_4 challenges created T_3 EHC. Biliary TH in the intestine may enter into circulation. However, TH challenges also increased intestinal TH metabolism. Thus, TH EHC may serve as an extrathyroidal source of TH, or TH metabolism may salvage I^-.

In conclusion, the intestine is more sensitive to increases in T_3, and it has mechanisms to prevent large increases in systemic T_3. However, intestinal TH metabolism, with peripheral metabolism, contributes to T_4 regulation by reducing the amount of T_4 absorbed from or returning to the intestine. Thus in rainbow trout, intestinal control of TH metabolism and the regulation of thyroidal status represents both a primitive intestinal control of T_3 and T_4 metabolism and absorption with a more derived central control of T_4.

List of Figures

Page

Page

Page

Page

Page

Page

Page

Page

Page

List of Tables

Page

Page

List of Abbreviations

ANS	8-anilo-1-napthelene sulfonic acid
ANOVA	Analysis of variance
BSA	Bovine serum albumin
°C	Celsius
Ci	Curie
D	Dark
d.f.	Degrees of freedom
DI	Distal intestine
DTT	Dithiolthreitol
EHC	Enterohepatic circulation
f	Femto
GSH	Glutathione
g	Gram; Force of gravity
I^-	Iodide
$*I^-$	Radioactive iodide
IRD	Inner-ring deiodination
IP	Iodoprotein
K_d	Enzyme mean apparent dissociation constant
K_m	Enzyme affinity (Michaelis-Menten Constant)
L	Liter; Light
L	Liver

MET	metabolism
MMI	Methylmercaptoimidazole
MI	Middle intestine
µ	Micro
m	Milli
min	Minute(s)
M	Molar
MS222	Tricaine methanesulfonate
n	Nano
NEI	Non-extractable iodoproteins
ORD	Outer-ring deiodination
ppm	Parts per million
p	Pico
PC	Pyloric caeca
RIA	Radioimmunoassay
rT_3	Reverse triiodothyronine
$*rT_3$	Radioactive reverse triiodothyronine
TRT	Treatment
T_2	Diiodothyronine
$*T_2$	Radioactive diiodothyronine
T_3	Triiodothyronine
$*T_3$	Radioactive Triiodothyronine
T_3-G	Glucuronidated triiodothyronine

T_4	Thyroxine
$*T_4$	Radioactive thyroxine
T_4-G	Glucuronidated thyroxine
TH	Thyroid hormone
*TH	Radioactive/radioiodine-labeled thyroid hormone
12L:12D	12-h light:12-h dark photoperiod
v/v	Volume/volume
V_{max}	Maximal velocity of enzyme reaction

Chapter 1

General Introduction

The thyroid gland, whether a discrete and encapsulated gland (mammals, birds, reptiles, and amphibians) or dispersed follicles (fish) couples iodide (I^-) from the blood with tyrosine to form 3',5',3,5-tetraiodothyronine (thyroxine, T_4) and triiodothyronine (T_3) (McNabb, 1992). T_4 is the main hormone released from the thyroid, yet 3',3,5-triiodothyronine (T_3) is the biologically more active form which binds with higher affinity to nuclear receptors and elicits physiological responses (Oppenheimer *et al.*, 1972). T_4 is converted outside of the thyroid to T_3 by deiodinase enzymes (Leonard and Visser, 1986).

Regulation of thyroidal status, or the availability of T_3 to bind receptors, is attributed to two main mechanisms of control: central and peripheral (Figure 1-1). The central control model, which is considered the main mechanism of control in mammalian studies, involves the hypothalamus as the central or master regulator, initiating the hypothalamus-pituitary-thyroid control of thyroid hormones (TH) released to circulation (McNabb, 1992; Eales and Brown, 1993). Negative feedback of TH at the hypothalamus and pituitary allows modulation of TH secreted.

In teleost fish, mainly T_4 and negligible amounts of T_3 are released from the thyroid (Chan and Eales, 1975; Eales 1979a; Grau *et al.*, 1986; Byamungu *et al.*, 1992). Only T_4 has been shown to have negative feedback effects at the hypothalamus (Peter 1971, 1972) and pituitary (Baker 1965, 1969a, b; Sage 1968; Sage and Bromage, 1970). Thus, central control is limited to the regulation of T_4 secretion from the thyroid, not T_3 availability (Eales and Brown, 1993; Kuhn, 1993; Eales, 1995).

Peripheral control, or the metabolism of T_4 and T_3 in the tissues, can contribute to the availability of T_3 to bind to receptors (McNabb, 1992). Since plasma T_3 often circulates at a higher concentration than T_4 (Chan and Eales, 1975; Eales, 1979a; Grau *et al.*, 1986; Byamungu *et al.*, 1992), peripheral control is considered the main regulator of T_3 availability in teleost fish (Eales, 1985). In peripheral tissues such as the liver, conversion of T_4 to T_3 by deiodination provides circulating T_3 (Leatherland, 1981; Pimlott and Eales, 1983; MacLatchy and Eales, 1993; Eales *et al.*, 1993) (Figure 1-2). With increased levels of T_3, hepatic tissues adjust circulating T_3 levels by removing the T_4 substrate (Sweeting and Eales, 1992b) or by removing the biologically active T_3 (MacLatchy and Eales, 1993). Thus, thyroidal status is maintained by deiodination based on the physiological state of peripheral tissues (Eales and Brown, 1993, Eales *et al.*, 1993). In addition to deiodination, other established and potential forms of TH metabolism include conjugation, deamination, ether-link cleavage, and covalent bonding to proteins (Van Middlesworth, 1974; DiStefano, 1988) (Figure 1-3). Clearance pathways such as urinary, branchial, biliary, and excretory pathways can also reduce available TH. Hence, peripheral tissues with access to TH and the ability to metabolize and clear TH may contribute to the regulation of thyroidal status. The intestine, known as an excretory pathway, may play a part in clearance of TH, but it is unknown if the tissue is capable of metabolizing TH.

The intestine has access to TH through the diet, the systemic blood, and the bile (Figure 1-4), yet the role of the intestine as a peripheral tissue contributing to the regulation of thyroidal status has not been studied extensively in any species. In lampreys (Eales *et al.*, 1997), hagfish (McLeese *et al.*, 2000), and sturgeon (Plohman *et al.*, 2002b),

several TH metabolism pathways were active, possibly contributing to thyroidal status. The only investigation into intestinal TH metabolism of in rainbow trout, was conducted by Law and Eales in 1973. In rainbow trout, the intestine demonstrated significant liberation of $^*I^-$, indicating the presence of TH metabolism pathways. Therefore, considering the mass of tissue and the possible influx of TH from the diet, the blood, or the bile, the intestine may provide a large pool of TH for metabolism and absorption to circulation. Thus, if TH metabolism (e.g. deiodination) occurs in the intestine, it may have a significant role in regulating thyroidal status.

Even though the intestine is mainly associated with an excretory role, it may actually serve as a dynamic pool of materials from which metabolism, temporary storage, and absorption take place. This conversion of TH and entry into circulation may comprise part of a TH enterohepatic circulation (EHC) which involves the cycling of substances from the blood to the liver to the bile to the intestine and back to the blood (Figure 1-4). Thus with EHC, the intestine is not solely considered an excretory route for TH. T_4 and T_3, can follow the EHC pathway, depending on the form of iodothyronine released into the bile and the extent of its absorption from the intestine. In mammals, biliary-excreted TH can contribute to the intestinal pool of TH (DiStefano, 1988; DiStefano *et al.*, 1992). From the intestine, unconjugated TH can be absorbed extensively and re-enter the enteric circulation (Briggs *et al.*, 1953; Chung and van Middlesworth, 1964; Sternlicht *et al.*, 1984; Hazenburg *et al.*, 1988; DiStefano, 1988; DiStefano *et al.*, 1988; Rutgers *et al.*, 1989; DiStefano *et al.*, 1992). Thus, a TH EHC is created, which may contribute to the regulation of whole-body TH (DiStefano *et al.*, 1993c). The same could be true for teleost fish.

There is limited knowledge of TH EHC in teleost fish. Previous studies have investigated individual components of TH EHC, and hence the potential for completion of the EHC, by evaluating intestinal access to TH through biliary supply and by evaluating reentry of TH to the plasma through absorption of TH from the intestine. There is abundant evidence for TH uptake by the liver and TH excretion into the bile (Sinclair and Eales, 1972; Eales and Sinclair, 1974; Finnson and Eales, 1996). Thus, the biliary route represents a source of TH released to the intestine, with more T_4 than T_3 delivered in its native, unconjugated form. Indirect evidence of intestinal absorption of TH has been documented in rainbow trout by measuring increased levels of plasma TH after ingestion of TH-supplemented diets (Higgs *et al.*, 1979; Higgs *et al.*, 1982; Cyr and Eales, 1990; Eales *et al.*, 1990; Eales and Finnson, 1991; Sweeting and Eales, 1992; Fines *et al.*, 1999) or injection of TH into the intestine (Collicutt and Eales, 1974; Eales and Sinclair, 1974; Whitaker and Eales, 1993). There was consistently a greater increase in plasma T_3 than T_4. These studies suggested that negligible cycling of TH occurred, since little TH or TH conjugates were absorbed from the intestine. Considering its tissue mass, the intestine cannot be ignored as a potentially large pool of TH for absorption to the circulation and EHC.

Prior to this research, the role of the intestine in the metabolism of TH and the regulation of thyroidal status in rainbow trout, *Oncorhynchus mykiss*, had not been addressed in a systematic manner. The selected literature review (Chapter 2) supports the view that the intestine serves as more than an excretory route for TH. The present study provides further support for the role of the intestine in TH regulation by: 1) characterizing intestinal TH metabolism pathways (Chapter 3); 2) measuring altered

activity of intestinal TH metabolism pathways after physiological challenges of chronic dietary T_4 and T_3 (Chapter 4); 3) measuring uptake of TH from the intestine into enterohepatic tissues (Chapter 5); and 4) measuring altered uptake of TH from the intestine into enterohepatic tissues after physiological challenges of chronic dietary T_4 and T_3 (Chapter 6). These investigations have helped to define the role of the intestine in TH metabolism and regulation of thyroidal status (Chapter 7).

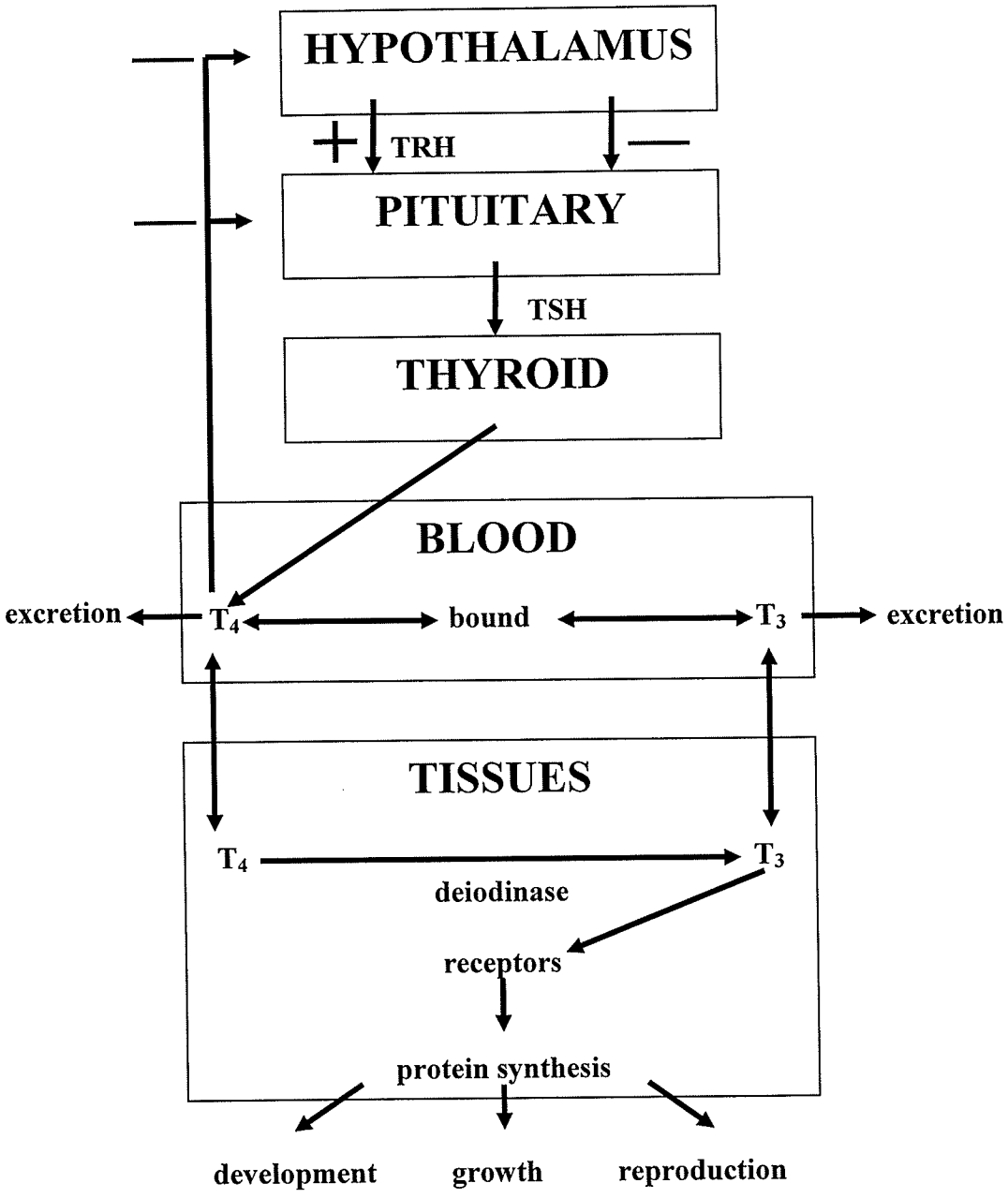


Figure 1-1. General thyroid model for teleost fish. The hypothalamus, through stimulatory pathways (+; thyrotropin releasing hormone, TRH) and inhibitory pathways (-), controls anterior pituitary secretion of thyrotropin (thyroid stimulating hormone, TSH). Thyroidal secretion of T_4 is stimulated by TSH. T_4 in the blood is mainly bound to plasma proteins, but the small free fraction exchanges with tissues. In the peripheral tissues, T_4 can be enzymatically deiodinated to T_3, which exchanges with the blood or it binds to receptors to elicit biological actions such as development, growth, and reproduction.

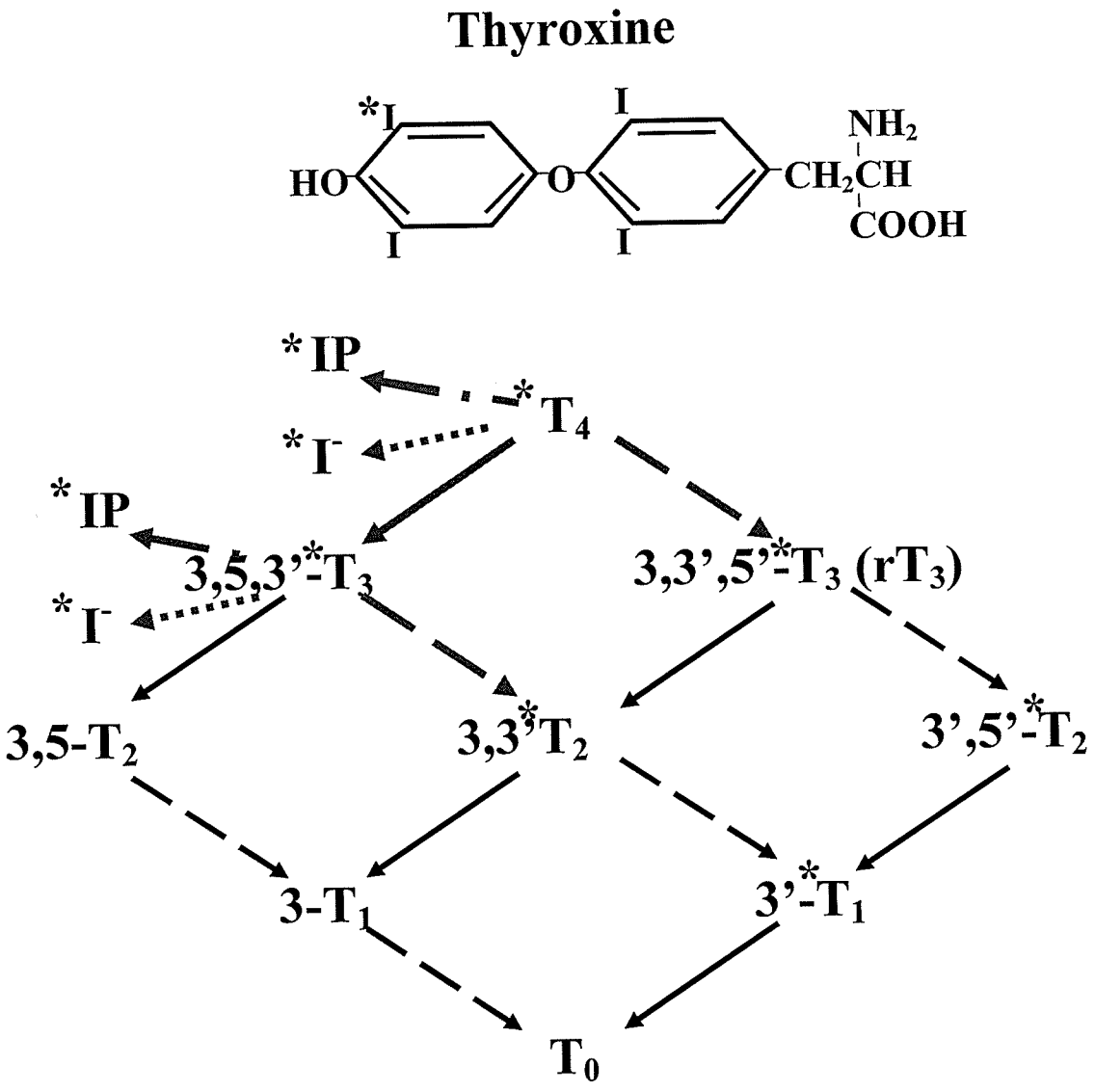


Figure 1-2. The structure of radiolabeled thyroxine (*T_4) with an asterisk indicating the site of ^{125}I on the outer ring. The pathways for outer-ring deiodination (ORD; ⟶) and inner-ring deiodination (IRD; ⟶) for T_4 are indicated. Additional TH metabolism pathways, such as T_4 complete deiodination (T_4cORD; ⇢), T_3ORD (⇢), T_4 iodoprotein production (T_4IP; ⟶), and T_3 iodoprotein production (T_3IP; ⟶) can also be followed by measuring radiolabeled products.

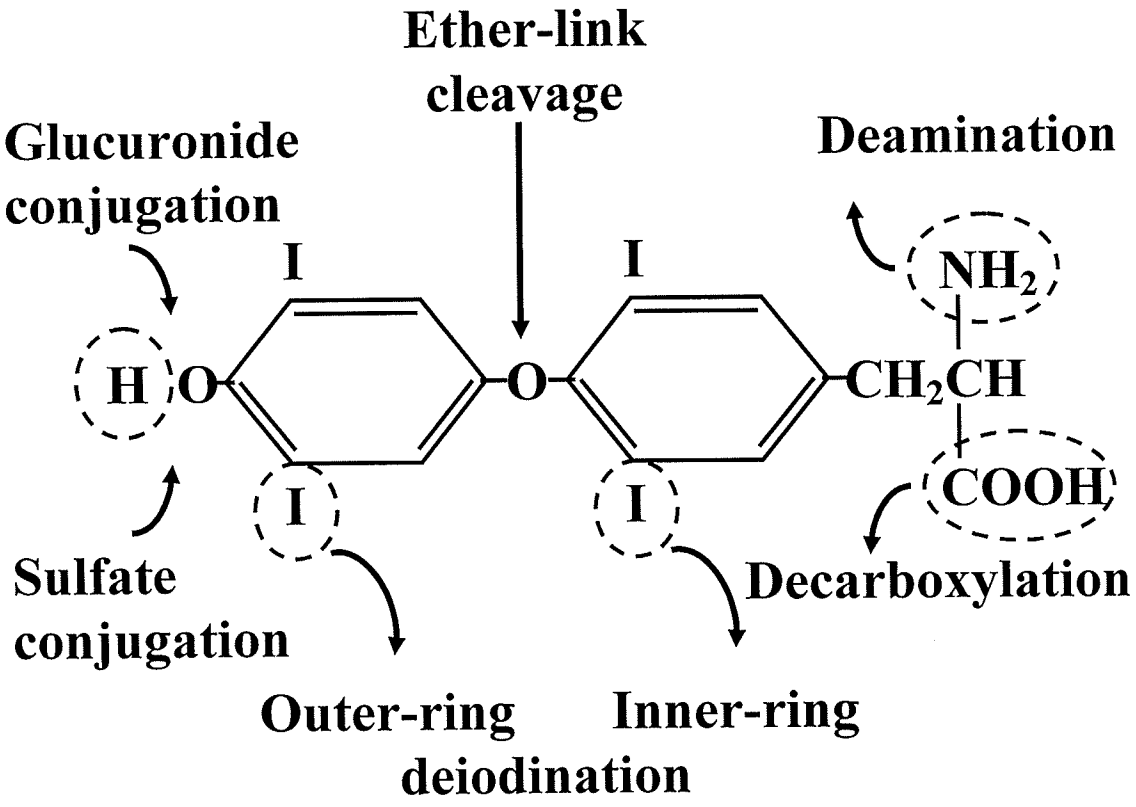


Figure 1-3. Possible pathways of TH metabolism including glucuronide conjugation, sulfate conjugation, outer-ring deiodination, inner-ring deiodination, decarboxylation, deamination, and ether-link cleavage.

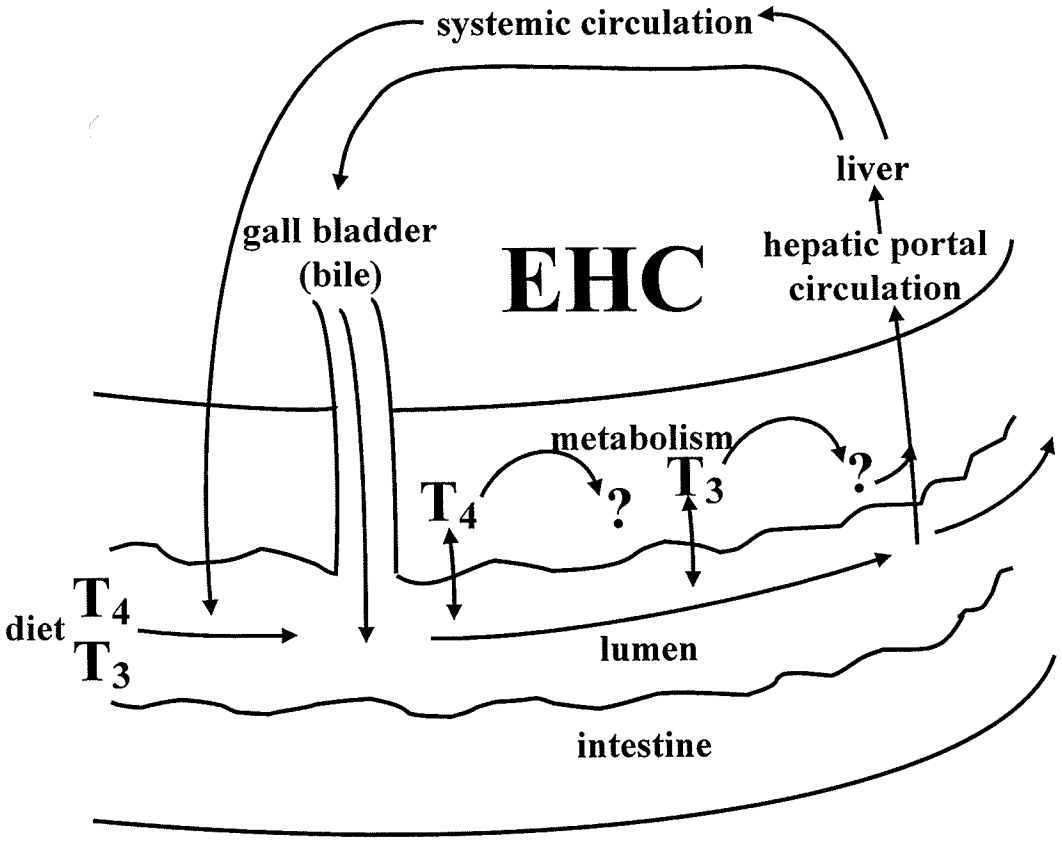


Figure 1-4. Diagram of sources of TH in the intestine, including the diet, bile, metabolism and systemic circulation, that may contribute to TH enterohepatic circulation (EHC) from the blood to the liver to the bile to the intestine and back to the blood.

Chapter 2

Selected Literature Review

This chapter reviews thyroid hormone (TH) regulation, TH metabolism (focusing on deiodination), enterohepatic circulation, and the role of the intestine in these processes for both higher vertebrates and teleost fish. The information for mammalian studies is more extensive than that found for teleost fish. The mammalian literature demonstrates the importance of the intestine in TH regulation through enterohepatic circulation, but there are limited studies on intestinal TH metabolism. There is less information on the role of the intestine in peripheral TH regulation and metabolism. In a few species of fish, there is information on intestinal TH metabolism. In teleost fish, there is some knowledge of TH biliary supply to the intestine and TH intestinal absorption. Hence, the potential role of the intestine as a peripheral regulator of thyroidal status is reviewed.

Overview of central control model for regulation of thyroid hormones

The thyroid system must be able to regulate thyroidal status, or the availability of 3',3,5-triiodothyronine (T_3), based on the physiological state of the animal. The regulation of thyroidal status is attributed to both a central control model and a peripheral control model. The central control model of TH regulation involves the hypothalamus as the central or master regulator (McNabb, 1992; Eales and Brown, 1993). In all vertebrates, the brain releases thyrotropin-releasing hormone (TRH) to stimulate thyrotropin (thyroid stimulating hormone, TSH) from the pituitary. TSH, the most important factor influencing function of the thyroid gland, stimulates almost all aspects of production and release of both 3',5',3,5-tetraiodothyronine (thyroxine, T_4) and T_3. In

mammals, T_4 accounts for 94% of the TH secreted by the thyroid, while T_3 accounts for the remainng 6% (McNabb, 1992). For teleost fish, T_3 has not been detected from the thyroid (Eales and Brown, 1993; Kuhn, 1993). Overall, T_4 is the main iodothyronine produced by the thyroid. T_4, which is considered a prohormone, is converted to T_3 in peripheral tissues, and it elicits physiological responses by binding to nuclear receptors in the target cell (Oppenheimer *et al.*, 1972).

In higher vertebrates, the main regulation of T_4 production, and T_3 conversion, is attributed to feedback control at the hypothalamus and the anterior pituitary. This central control of the hypothalamic-pituitary-thyroid axis is based on circulating free TH. The hypothalamic release of TRH is negatively controlled by feedback from circulating TSH and TH (Spira and Gordon, 1986). At the anterior pituitary, TSH is negatively controlled by feedback from TH (Spira *et al.*, 1979). Circulating T_3 and T_3 produced locally from T_4 decrease the production of TSH α and ß subunits (Chin *et al.*, 1985; Shupnik *et al.*, 1989) and the release of TSH (Tong, 1974). Thus, central control in higher vertebrates is regulated by T_3, which in turn regulates T_3 availability.

In teleost fish, central control mechanisms also include hypothalamic control of TSH release from the pituitary (Peter and McKeown, 1975; Bromage *et al.*, 1976; Peter and Fryer, 1983; Dickhoff and Darling, 1983; Eales and Himick, 1991) and TSH-stimulated release of T_4 from the thyroid (Chan and Eales, 1975, 1976; Brown *et al.*, 1978; Milne and Leatherland, 1980; Ng *et al.*, 1982; Swanson *et al.*, 1988; Inui *et al.*, 1989; Grau *et al.*, 1986). T_4-mediated, not T_3-mediated, negative feedback at the hypothalamus (Peter, 1971, 1972) and pituitary (Baker, 1965, 1969a, b; Sage, 1968; Sage and Bromage, 1970) regulates the central control mechanism. Thus, central control is

limited to the regulation of T_4 secretion from the thyroid, not T_3 availability (Eales and Brown, 1993; Kuhn, 1993; Eales, 1995).

Overview of peripheral control model for regulation of thyroid hormones in teleosts

More emphasis is placed on peripheral regulation of TH than central regulation in teleost fish. In the central control model, levels of thyroid hormones available for binding to the nuclear receptors in tissues were modulated by the brain and pituitary. In the peripheral control model, immediate requirements of TH by the active tissues are regulated by the tissues themselves.

In teleost fish, plasma T_4 and T_3 regulation are largely independent of each other (Grau *et al.*, 1986; Eales and Himick, 1991; Eales and Brown, 1993). Plasma T_3 often circulates at a higher concentration than T_4, yet only T_4 is secreted by the thyroid (Chan and Eales, 1975; Eales, 1979a; Grau *et al.*, 1986; Byamungu *et al.*, 1992). In addition, plasma T_4 (Baker, 1965; Peter, 1971), not plasma T_3 (Eales *et al.*, 1990), suppresses TSH release. Thus, central control determines the plasma T_4 levels. Peripheral control, in contrast, regulates T_3 availability.

Evidence supports peripheral control as the primary control of thyroidal status in teleost fish. A lack of correlation between plasma T_4 and plasma T_3 is demonstrated when TSH or T_4 challenges do not elevate plasma T_3 levels. TSH injection results in an increase in plasma T_4, yet plasma T_3 levels remain unaffected (Chan and Eales, 1975; Brown *et al.*, 1978; Milne and Leatherland, 1978). Additionally, T_4 challenges do not significantly change plasma T_3 levels (Fok and Eales, 1984; Swanson *et al.*, 1988; Kuhn, 1993). These studies support extrathyroidal regulation of T_3 availability.

With peripheral control, changes in circulating TH would not result in a change in thyroidal output as seen in central control. Instead, peripheral autoregulation of T_3 is controlled by TH metabolism. There are many forms of TH metabolism that can contribute to peripheral control of TH (see Overview of TH metabolism and Figure 1-3). Deiodination, the step-wise remove of I^- from iodothyronines (see Deiodination in mammals and Figure 1-2), is a main contributor to peripheral control of T_3 availability, especially in the liver. For example, a dose-dependent increase in plasma T_3 results in a decrease in the conversion of T_4 to T_3 through a decrease in hepatic T_4 outer-ring deiodination (T_4ORD) (Eales *et al.*, 1990). Increased plasma T_3 through dietary supplementation also decreases hepatic high-K_m (low substrate affinity) T_4ORD while increasing hepatic T_4 inner-ring deiodination (T_4IRD), which converts T_4 to inactive reverse T_3 (rT_3), and T_3 inner-ring deiodination (T_3IRD), which converts T_3 to T_2 (Eales and Finnson, 1991; Sweeting and Eales, 1992a; MacLatchy and Eales, 1993). In addition, low-K_m T_4ORD in the gill and high-K_m T_4ORD in the kidney are inhibited. At the cellular level, direct T_3 autoregulation of T_4ORD in hepatocytes has been demonstrated to maintain T_3 homeostasis (Sweeting and Eales, 1992b). Thus, various deiodinase pathways are coordinated in the trout to maintain a set point for T_3 levels available as demonstrated by a decrease in T_3 formation and degradation of excess T_3 following increased plasma T_3 levels.

Peripheral control may not only contribute to plasma pools of T_3 but also derive T_3 for local tissue use. For example, teleost fish contain both a high-K_m (10 nM substrate level; low enzyme-substrate affinity) and a low-K_m (0.1 nM substrate level; high enzyme-substrate affinity) T_4ORD that converts T_4 to T_3. Liver contains both isozymes, while

gills have low-K_m isozymes and kidneys have high-K_m isozymes (MacLatchy and Eales, 1992b). The different substrate affinities may be attributed to different roles of the enzymes. A high-K_m isozyme requiring high T_4 substrate levels, such as the one detected in liver and kidney (Leatherland, 1981; Flett and Leatherland, 1989; MacLatchy and Eales, 1992b), is correlated with the plasma as the source of hormone. The greater the amount of T_3 derived from circulation, the more predominant the high-K_m isozyme. Hence the liver, which has both high- and low-K_m isozymes, derives 50% of intracellular T_3 from plasma T_4. The kidney only has a high-K_m isozyme, and 72% of receptor-bound T_3 is derived from plasma. Conversely, gills only have low-K_m enzymes, or high enzyme-substrate affinity, which functions at the low substrate level predicted to exist intracellularly. Thus, 28% of receptor-bound T_3 is from the plasma (Eales *et al.*, 1993).

With this metabolism, peripheral tissues are able to maintain T_3 availability and maintain T_3 homeostasis both in the plasma and in the tissues. Hence, peripheral tissues with deiodinating activity and access to circulating thyroid hormones exert a role in regulation of thyroid hormone metabolism and thyroidal status.

Overview of thyroid hormone metabolism

There are many types of TH metabolism that can occur, and specific pathways of metabolism include ether-link cleavage, conjugation, deamination, decarboxylation, covalent binding to proteins, and deiodination (Van Middlesworth, 1974; Burger, 1986; DiStefano, 1988) (Figure 1-3). Of these, ether-link cleavage of T_4 or T_3, which results in an unstable quinone and 3,5-diiodotyrosine after breaking the ether-link bond, has not been detected in teleost fish (Visser, 1990).

Side chain modification includes deamination and oxidative decarboxylation of the alanine side chain resulting in acetic acid analogs such as 3,5,3',5'-tetrathyroacetic acid (tetrac) and 3,5,3'-triiodothyroacetic acid (triac) (Burger, 1986).

Conjugation, which can include sulfation or glucuronidation is the addition of sulfate or glucuronic acid, respectively, to the phenolic hydroxyl group. These reactions increase water-solubility of TH which enhances their excretion in bile and urine (Visser, 1990). Glucuronidation is the initial step in enterohepatic circulation of thyroid hormones (DiStefano, 1988), and T_4 is the main iodothyronine involved (Gershengorn *et al.*, 1987). Glucuronidated TH are excreted in the bile and then enter the intestine (Visser, 1990). However, these conjugates can be deconjugated by bacteria in the intestine, and liberated iodothyronines can be reabsorbed (DiStefano, 1988). Therefore, glucuronidation does not necessarily lead to an irreversible loss of thyroid hormones. In contrast, sulfated thyroid hormones, primarily T_3, are not detected in the bile of rats (Sekura *et al.*, 1981). Hepatic sulfation accelerates deiodination of most iodothyronines, metabolizing them before entry into bile (Visser, 1994; Visser *et al.*, 1990).

In teleost fish, glucuronidated TH have been detected in marine plaice, brook trout, and rainbow trout (Sinclair and Eales, 1972; Collicutt and Eales, 1974; Finnson and Eales, 1997). Hepatic glucuronidation of TH occurs by more than one isozyme in trout. TH are eliminated by conjugation and entry into the bile, since they are neither deconjugated nor deiodinated by trout liver (Finnson *et al.*, 1999) In teleost fish, sulfation of TH occurs in the liver ($rT_3 > T_4 = T_3$), however, deiodination of these sulfates is negligible (Finnson and Eales, 1998; Finnson *et al.*, 1999). Unlike mammals, sulfation does not lead to a loss or excretion of TH; it may allow cellular storage of TH.

Iodothyronines have been documented to be covalently bonded to protein, resulting in non-extractable iodoproteins (NEI). Interest in NEI peaked during 1970's when they were detected in the thyroid, plasma, mammary gland, salivary gland, and stomach. The protein component may be thyroglobulin or albumin, but the exact nature of the protein is not always known. The actual stucture of these NEI has not been determined. Futhermore, biological properties of the NEI have not been assessed (Ui, 1974).

Deiodination is a stepwise and sequential removal of iodide (I^-) from iodothyronines (Figure 1-2). Several different isozymes have been detected in tissues that can remove I^- from the outer ring or the inner ring of the iodothyronines. Deiodination can activate or inactivate iodothyronines. For example, outer-ring deiodination can produce the biologically active form, T_3, from T_4, or the activation of T_4 can be prevented by inner-ring removal of I^-, resulting in the biologically inactive iodothyronine, rT_3.

As mentioned, metabolism of iodothyronines through any pathway can play an important function in overall thyroid hormone regulation. For example, deiodination can increase plasma T_3 available for tissues. It can also increase cellular T_3 centrally in the brain or peripheral tissues, adding to both central and peripheral control.

Deiodination in mammals

In mammals, stepwise removal of I^- is performed by at least three deiodinase isozymes, type I, II, and III. These isozymes remove either outer-ring iodide or inner-ring I^-. They also vary with respect to tissue location, substrate preference, substrate affinity, interaction with thiol cofactors, and interaction with inhibitors (Leonard and Visser,

1986). In rats and human, 80% of T_4 is degraded extrathyroidally by outer-ring deiodinases.

Type I isozyme, found in liver, kidney, and thyroid, is capable of outer- and inner-ring deiodination (ORD and IRD, respectively). It converts T_4 to T_3 by outer-ring removal of iodine, or it can inactivate T_4 to rT_3 through inner-ring deiodination (Hesch and Kohrle, 1986). Studies on T_4 as the substrate suggest that type I deiodinase contributes to plasma T_3 levels (Crantz *et al.*, 1982). Even though it was first recognized for activating T_4, rT_3 is its preferred substrate, and the main role of type I deiodinase may be to dispose of rT_3. Hence rT_3 can compete with T_4 as a substrate, inhibiting T_3 production (Hesch and Koehrle, 1986). Kinetic studies of the enzyme reveal a high-K_m enzyme (approximately 1 μM; low substrate affinity) that is stimulated by thiols, exhibits ping-pong kinetics, and is inhibited by propylthiouracil (PTU), iodoacetate (IA), and iopanioic acid (IPA).

Type II isozyme is also capable of outer-ring conversion of T_4 to T_3. However, it is located in the central nervous system, pituitary, brown adipose tissue, placenta, and skin. In addition, it is a low-K_m isozyme (approximately 1 nM; high substrate affinity), which correlates with suspected cellular T_4 levels. Thus, it has been speculated that type II deiodinase is mainly involved in central regulation as an internal indicator of thyroidal status (Hesch and Koehrle, 1986). Type II is stimulated by thiols, exhibits sequential kinetics, is not affected by PTU, but is inhibited by IA and IPA.

Type III isozyme is an inner-ring deiodinase found in the central nervous system and placenta. It prefers T_3 as a substrate over T_4, so it is considered a deactivating isozyme by degrading T_3 to 3,3'-T_2 and T_4 to rT_3 (Leonard and Visser, 1986). It is a low-

K_m isozyme (approximately 40 nM) that is stimulated by thiols through sequential kinetics. PTU has no effect, but IA and IPA are inhibitory.

Role of the intestine in deiodination in higher vertebrates

Few studies have characterized intestinal TH metabolizing capabilities. Of the few studies, a comparison was made of intestinal deiodination to hepatic deiodination. Galton *et al.* (1991) measured rat intestinal TH metabolism. ORD activity was highest in the fetal intestine and very low in the adult intestine, whereas the opposite was true for liver ORD activity. IRD activity was also present in the intestine. Suvarna *et al.* (1993) measured intestinal ORD activity in chickens. It was similar in biochemical characteristics to hepatic ORD activity in mammals (Leonard and Visser, 1986) and birds (Freeman and McNabb, 1991; McNabb *et al.*, 1991; Darras *et al.*, 1992). In adult chickens, intestinal ORD was lower than hepatic ORD, but it was higher than activity measured in rats.

Deiodination in teleosts

In teleost fish, several isozymes exist, but they differ in characteristics from mammalian isozymes. Since deiodination plays a crucial role in peripheral control of thyroid hormone, T_4ORD is important in controlling thyroidal status by regulating T_3 levels for receptor binding.

In the liver, a high-K_m (approximately 10 nM) and a low-K_m (approximately 0.1 nM) T_4ORD converts T_4 to T_3. The different substrate affinities may determine different roles of the T_4ORD's. The high-K_m isozyme may supply plasma T_3 levels, while the low-K_m isozyme supplies intracellular T_3 (Eales *et al.*, 1993). An inner-ring deiodinase is also located in the liver, however its activity is only induced under stress or increased plasma

T_3 (Eales *et al.*, 1993). In the kidney, a high-K_m T_4ORD has been detected, while a low-K_m T_4ORD is reported in the gills.

Role of the intestine in deiodination in fish

Intestinal deiodination has been measured in the intestine of a few species of fish. Intestinal deiodination activities in the Atlantic cod (Cyr *et al.*, 1997) and American plaice (Adams *et al.*, 2000) were low. In sturgeon, activities of intestinal deiodination pathways (T_4ORD, T_4IRD, T_3IRD, and rT_3IRD) were significant, and in this species, T_4ORD may contribute to plasma T_3. However, intestinal deiodination was lower than hepatic deiodination (Plohman *et al.*, 2002). In lampreys (Eales *et al.*, 1997, 1999), T_4ORD was active in the intestine. T_4IRD and T_3IRD were negligible in the larval intestine but present in that of the upstream-migrant lampreys. In hagfish (McLeese *et al.*, 2000), intestinal T_4ORD, T_4IRD, and T_3IRD were active. In both the lamprey and the hagfish, intestinal deiodination (T_4ORD) predominated over hepatic deiodination. Thus, intestinal deiodination may represent ancestral TH metabolism pathways that are actively retained in more "primitive" fish lineages but still present in teleost fish.

As far as intestinal metabolism of TH of teleosts is concerned, a preliminary study evaluated the deiodinating activity through trichloroacetic acid (TCA) precipitation of protein-bound radiothyronines and thin layer chromatography (TLC) separation of products. These techniques measured deiodination of T_4 double-labeled with *I and incubated with whole tissue homogenates (Law and Eales, 1973). Even without dithiolthreitol as an enzyme cofactor, deiodinase or other enzyme activity was detected by the presence of free $*I^-$ not precipitated by TCA and by peaks of radiolabeled-products on

TLC. Thus, these results indicate the breakdown of TH and the occurrence of TH metabolism in the intestine.

Importance of iodide availability

The metabolism of TH liberates I^-, which can be recycled for the synthesis of TH in the thyroid. Other sources of I^- include intestinal transport from food or gill transport from water. There are varying amounts of I^- (5 μg/dL in seawater; 1 μg/dL to 0.01 μg/dL in freshwater) (Eales, 1979). Yet, freshwater teleosts (salmonids) may attain levels of plasma I^- ranging from 100 to 200 μg/dL which exceed environmental levels (Leloup, 1970; Gregory and Eales, 1975).

The branchial and intestine I^- transporters (presumably Na^+/I^- symporters) are quite efficient at maintaining plasma I^- levels (Hunn and Fromm, 1966; Leloup, 1970; Gregory and Eales, 1975). Even when subjected to low dietary I^- levels, the branchial I^- pump was able to elevate plasma I^- over normal levels (Higgs and Eales, 1971).

Unlike mammals, certain teleosts exhibit a plasma pre-albumin protein which non-covalently and reversibly binds I^- (Leloup, 1970). This protein contributes to high plasma I^- levels by reducing filtration loss of I^- at renal, gill, integumentary or gut exchanges surfaces (Gregory and Eales, 1975).

Another difference from mammals is the physiological response of rainbow trout to elevated I^- levels. In humans and rats, the Wolff-Chaikoff effect occurs when an acute excess of I^- inhibits thyroidal secretion (DeGroot and Stanbury, 1975). In rainbow trout, acute I^- excess results in an increase of plasma T_4 (Eales *et al.*, 1986a).

Overall, the rainbow trout in its low-I^-, freshwater environment has the ability to obtain or conserve I^-, ensuring TH production.

Role of cofactors in deiodination

Thiol cofactors such as dithiolthreitol (DTT), dihydrolipoamide, or monoglutathione (GSH) provide an exogenous thiol that significantly increases trout microsomal deiodination activity in the liver (Pimlott and Eales, 1983; Shields and Eales, 1986). Sulfhydryl groups or selenocysteine residues are found at the deiodinase enzyme catalytic site in mammals (Visser, 1979; Kohrle, 1996). Thiols can reduce the deiodinase enzyme, forming a catalytically active enzyme. The exogenous thiol cofactor is believed to replace an endogenous biological equivalent. In mammals, GSH is an effective thiol cofactor for ORD in intestinal tissues (Galton *et al.*, 1991) and hepatic and kidney tissues (Goswami and Rosenberg, 1988). In rainbow trout microsomal assays, DTT is effective as a cofactor (Eales *et al.*, 1993). The exogenous thiol cofactor is believed to replace an endogenous biological equivalent. In fact, Galton *et al.*, (1991) suggested that GSH may be an activator of ORD *in vivo*. The nature of an endogenous cofactor in fish is not yet understood.

I*n vivo*, deiodinase enzymes may not be regenerated by thiol cofactor (Kohrle *et al.*, 1993). Binding and deiodination may promote enzyme tranlocation and degradation (St. Germain and Croteau, 1989).

Role of the intestine in thyroid hormone enterohepatic circulation in higher vertebrates

In mammals, the intestine has an extensive role in enterohepatic circulation which cycles THs from the liver to the intestine and back to the liver. Transport of TH from the liver to the intestine includes mesenteric arterial or biliary supply (Briggs *et al.*, 1953; Chung and van Middlesworth, 1964; Sternlicht *et al.*, 1984; Hazenburg *et al.*, 1988;

DiStefano, 1988). TH entering the intestine can be conjugated, whereby can sulfate or glucuronide replaces the phenolic hydroxyl, or unconjugated, intact form (DiStefano, 1988). In the bile, 75% of TH are conjugated. Of those conjugated, approximately 91% are glucuronidated and 9% are sulfated (DiStefano, 1988). Transit time for TH in the intestine and possible binding of the hormones allow for temporary storage of the hormone in the intestine (Chung and van Middlesworth, 1964, DiStefano, 1988). Some of these hormones remain bound to luminal contents, hence the intestine is an excretory pathway (Chung and van Middlesworth, 1964). However, unbound hormone can be absorbed from the intestine, providing another source of TH for possible biological actions (Albert *et al.*, 1952; Albert and Keating, 1952; Chung and van Middlesworth, 1964; Cottle and Veress, 1965; DiStefano *et al.*, 1992).

Besides providing a source of hormone from luminal storage, the intestinal tissue itself may regulate metabolism of TH, as mentioned previously. In addition, bacteria in the intestine are capable of binding and oxidatively degrading T_4 and T_3 (DiStefano *et al.*, 1993b) The bacteria are also known to deconjugate glucuronidated or sulfated TH (Taurog *et al.*, 1952; Williams *et al.*, 1965; de Herder *et al.*, 1985, 1986; Hazenburg *et al.*, 1988; Rutgers *et al.*, 1989; Nguyen *et al.*, 1993; DiStefano *et al.*, 1993a). Conjugation of TH decreases the absorptive properties of the hormones from the intestine. Deconjugation by bacteria may allow further deiodination to occur as a clearance route and an I^- salvaging mechanism, or it may allow absorption from the intestine. Depending on the luminal pool of hormones, metabolism, and uptake of hormones, the intestine could provide a significant source of TH and I^-.

Role of the intestine in thyroid hormone enterohepatic circulation in teleosts.

There is limited knowledge of TH EHC in teleost fish. Previous studies have investigated individual components of TH EHC, and hence the potential for completion of the EHC, by evaluating intestinal access to TH through biliary supply and by evaluating reentry of TH to plasma through absorption of TH from the intestine. The fish intestine may, therefore, contribute to the regulation of thyroidal status if TH discharged from bile or obtained through the diet is absorbed from the intestine and contributes to available systemic TH.

In salmonids and other teleost fish, there is abundant evidence for TH uptake by the liver and TH excretion into the bile. Marine plaice, *Pleuronectes platessa* (Osborn and Simpson, 1969), Atlantic salmon, *Salmo salar* (Eales, 1969), brook trout, *Salvelinus fontinalis* (Eales, 1969, 1970; Eales *et al.*, 1971; Sinclair and Eales, 1972; Eales and Sinclair, 1974), rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), brown bullhead (*I. nebulosus*), black bullhead (*I. melas*), burbot (*Lota lota*), goldeye (*Hiodon alosoides*), freshwater drum (*Aplodinotus grunniens*), white suckers (*Catastomus commersoni*), and hornyhead chub (*Nacomis biguttatus*) accumulated T_4 or T_3 in the bile (Sinclair and Eales, 1972).

Experiments to determine the forms and amounts of TH accumulated in the bile have followed the fate of administered *TH. After an intraperitoneal injection of $*T_4$ into fasted brook trout, over 50% of the radioactivity accumulated in the bile was radiolabeled $*T_4$ ($*T_4$) (Sinclair and Eales, 1972). After an intraperitoneal $*T_4$ injection into rainbow trout, $*T_4$ and its less absorbable conjugates were also accumulated in the bile after 24 h. After a similar injection of $*T_3$, mainly $*T_3$ conjugates (rather than $*T_3$) were

accumulated in bile of rainbow trout (Finnson and Eales, 1996). Furthermore, feeding brook trout caused the rapid release of bile into the intestine (Eales and Sinclair, 1974). Thus in salmonid fish studied to date, the biliary route represents a source of TH released to the intestine, with more T_4 than T_3 delivered in its native, unconjugated form.

There is far less information for teleost fish on the completion of EHC with TH uptake from the intestine. Just as the intestine is a main site for absorption of nutrients such as amino acids, glucose, and fatty acids, it would make sense that it is also the site for absorption of amino acid derivatives such as TH. The absorption of TH from the intestine has been evaluated through changes in physiological parameters attributed to TH actions and through measurements of increased plasma TH levels. Feeding T_3 to fish has promoted effects such as growth, food consumption, and food conversion that are attributed to TH actions (Higgs *et al.*, 1979; Woo *et al.*, 1991; Moon *et al.*, 1994). T_4, at higher levels, had limited effects on food consumption (Higgs *et al.*, 1979). Indirect evidence of intestinal absorption of TH has been documented in rainbow trout by measuring increased levels of plasma TH after ingestion of TH-supplemented diets (Higgs *et al.*, 1979; Higgs *et al.*, 1982; Cyr and Eales, 1990; Eales *et al.*, 1990; Eales and Finnson, 1991; Sweeting and Eales, 1992b; Fines *et al.*, 1999). There was consistently a greater increase in plasma T_3 than T_4 with the same dietary TH dose.

More direct measurements of TH uptake from the intestine have been made by following *TH injected into the lumen of a fish intestine. In starved channel catfish, 20-25% of the radioactivity associated with $*T_4$ or $*T_4$ and its glucuronidated form ($*T_4$-G) were absorbed after 24 h from the lumen of a ligatured middle intestine (Collicutt and Eales, 1974). In brook trout, between 12-36% of the radioactivity associated with $*T_4$ or

*T_4 and *T_4-G injected into a double-ligatured intestinal loop of middle intestine and distal intestine was lost from the intestinal lumen after 24 h (Eales and Sinclair, 1974). When starved rainbow trout were injected with *T_4 and then fed, the biliary accumulated *T_4 and *T_4-G were released to the intestine, but there was no increase in plasma *T_4 relative to unfed controls (Eales and Sinclair, 1974). This suggested that negligible cycling of TH occurred, since little TH or TH conjugates were absorbed from the intestine in comparison to the amount returning to the intestine. Thus in teleost fish, the intestine is traditionally considered an excretory pathway for TH. Though, considering its mass of tissue, the intestine cannot be ignored as a possible large pool of TH for absorption to the circulation and EHC.

Based on the above, and since a given level of dietary T_3 increased circulating T_3 levels to a greater degree than dietary T_4 increased circulating T_4 levels (Higgs *et al.*, 1979, 1982), it is believed that T_4 was more poorly absorbed than T_3 from the digestive tract. A study by Whitaker and Eales (1993) was conducted to compare T_4 and T_3 absorption from the intestinal lumen of rainbow trout. Large doses of *T_4 or *T_3 were injected into the middle intestine, via an anal catheter, and uptake was measured over a period of 48 h. In agreement with the above studies, T_3 was removed from the intestine and entered blood and tissues more extensively than T_4.

In summary, there is considerable evidence that high proportions of TH are secreted in fish bile. It has been concluded that significant biliary excretion of unconjugated TH occurs, mainly in the form of T_4. There is far less information on what appears to be limited uptake from the intestine. However, the existing data suggest that,

at least in salmonid fish, a higher proportion of T_3 than T_4 can enter the circulation from the intestinal lumen.

Chapter 3

In vitro Thyroid Hormone Metabolism and Assay Conditions for Intestinal Metabolism of the Thyroid Hormones, 3',5',3,5-tetraiodothyronine and 3',3,5-triiodothyronine, in Rainbow Trout

Introduction

In teleost fish, the thyroid gland iodinates and couples tyrosines to form 3',5',3,5-tetraiodothyronine (thyroxine, T_4). T_4 is the main hormone released from the thyroid, yet 3',3,5-triiodothyronine (T_3) is the biologically more active form which binds with higher affinity to nuclear receptors and elicits physiological responses. The regulation of plasma T_4 level is attributed to central control mechanisms which include hypothalamic control of thyroid stimulating hormone (TSH) release from the pituitary (Peter and McKeown, 1975; Bromage *et al.*, 1976; Peter and Fryer, 1983; Dickhoff and Darling, 1983; Eales and Himick, 1991), TSH-stimulated release of T_4 from the thyroid (Chan and Eales, 1975, 1976; Brown *et al.*, 1978; Milne and Leatherland, 1980; Ng *et al.*, 1982; Swanson *et al.*, 1988; Inui *et al.*, 1989; Grau *et al.*, 1986), and T_4 negative feedback at the hypothalamus and pituitary (Peter, 1971, 1972; Baker, 1965, 1969a,b; Sage, 1968; Sage and Bromage, 1970). However, plasma T_3 often circulates at a higher concentration than T_4 (Chan and Eales, 1975; Eales, 1979a; Grau *et al.*, 1986; Byamungu *et al.*, 1992), and plasma T_4 and plasma T_3 regulation are largely independent of each other (Grau *et al.*, 1986; Eales and Himick, 1991; Eales and Brown, 1993). In peripheral tissues, such as the liver, conversion of T_4 to T_3 by deiodination provides circulating T_3 (Leatherland, 1981; Pimlott and Eales, 1983; MacLatchy and Eales, 1993; Eales *et al.*, 1993). This

deiodination and other thyroid hormone (TH) metabolism pathways determine T_3 homeostasis. Thus, peripheral metabolism is deemed important in regulating thyroidal status, or the availability of T_3 to bind to receptors, in teleost fish.

In rainbow trout, the main control of T_3 homeostasis is deiodination in peripheral tissues (Eales *et al.*, 1993; Eales, 1995; Figure 1-2). The liver is a main contributor to regulation of thyroidal status. It exhibits outer-ring deiodination (ORD) and inner-ring deiodination (IRD) pathways, including T_4ORD, T_4IRD, and T_3IRD, that work together to autoregulate T_3 intracellularly and to provide T_3 for other tissues that may primarily derive their source of T_3 from the plasma pool (Eales *et al.*, 1993). Other established and potential forms of TH metabolism include conjugation, deamination, ether-link cleavage, and covalent bonding to proteins (Van Middlesworth, 1974; Hennemann, 1986; DiStefano, 1988; Figure 1-3). Tissues other than the liver may contribute to T_3 homeostasis and the plasma pool of T_3 if they have access to a large source of T_4 and have metabolizing capabilities such as deiodination.

The role of the intestine as a peripheral tissue contributing to the regulation of thyroidal status has not been studied extensively in any species. The intestine has access to TH through the diet, the systemic circulation, and the bile (Figure 1-4). However, few studies have characterized intestinal TH metabolizing capabilities. In the few studies conducted to date, a comparison to hepatic deiodination has usually been made. Galton *et al.* (1991) measured rat intestinal TH metabolism and reported that ORD activity was highest in the fetal intestine but very low in the adult intestine, whereas the opposite was true for liver ORD activity. IRD activity was also present in the intestine. Survarna *et al.* (1993) measured intestinal ORD activity in chickens, and it was similar in biochemical

characteristics to hepatic ORD activity in mammals (Leonard and Visser, 1986) and birds (Freeman and McNabb, 1991; McNabb *et al.*, 1991; Darras *et al.*, 1992). In adult chickens, intestinal ORD was lower than hepatic ORD, but it was higher than activity measured in rats. In juvenile and adult sea lamprey, T_4ORD activity was greater in the intestine than the liver (Eales *et al.*, 1997). In hagfish, T_4ORD, T_4IRD, and T_3IRD could be measured (McLeese *et al.*, 2000). Similar to lampreys, intestinal T_4ORD activity greatly exceeded that of liver. In lake sturgeon, Plohman *et al.* (2001) measured T_4ORD, T_4IRD, T_3IRD, and reverse T_3ORD (rT_3ORD) activities in liver and intestine and showed that the intestine may contribute significantly to plasma T_3 levels and rT_3 deiodination. The only investigation into intestinal TH metabolism in rainbow trout was conducted by Law and Eales in 1973. Precipitation with trichloroacetic acid (TCA) and identification of products using thin-layer chromatography (TLC) was used to measure deiodination of T_4 double-labeled with radioactive iodide (*I) and incubated with whole-tissue homogentates. The intestine demonstrated significant liberation of *I, indicating the presence of TH metabolism pathways. Therefore, considering the mass of tissue and the possible influx of TH from the diet, blood, or bile, the intestine may provide a large pool of TH for metabolism and absorption to circulation. Thus, if TH metabolism, such as deiodination, occurs in the intestine, it may have a significant role in regulating thyroidal status.

This chapter reports investigations on the types of TH metabolism pathways that occurred in the various regions of the trout intestine, and it defined the conditions for *in vitro* measurement of these pathways using microsomal assays. The regions of the intestine investigated for TH metabolism were the pyloric caeca (PC), middle intestine

(MI), and distal intestine (DI). Liver TH metabolism was also measured as a reference. Liver TH metabolism pathways in rainbow trout were known, and the *in vitro* microsomal assay conditions were previously established. Activities of the TH metabolism pathways were measured using LH-20 column chromatography, protein precipitation, and high-performance liquid chromatography (HPLC). Determination of the assay conditions for the various TH metabolism pathways involved examination of cofactor concentration, incubation time, and pH. Demonstration of TH metabolism pathways in the intestine would define a potential role of the intestine in contributing to thyroidal status by regulating the availability of TH to enter into circulation or to be excreted.

Materials and Methods

Fish maintenance

Rainbow trout were obtained from the Rockwood Hatchery (Gunton, Manitoba) and held at the University of Manitoba in running, aerated, dechlorinated, City of Winnipeg water at 12°C, on a 12-h light:12-h dark (12L:12D) photoperiod. Sexually immature trout (200-500 g) were fed 1% of body mass of commercial trout feed diet (Martin Feed Mills, Elmira, Ontario; 3.2 mm diameter; manufacturer's analysis: protein 42%, crude fat 16%, fiber 3%, sodium 0.35%, calcium 1%, phosphorus 0.7%, vitamin A 7500 IU/kg, vitamin C 180 IU/kg, vitamin D_3 2500 IU/kg, vitamin E 95 IU/kg) once daily at 9 AM.

Sampling

Fish were anesthetized with 0.067 g/L of tricaine methanesulfonate (MS222; Syndel Laboratories, Vancover), and killed by a blow to the head. The liver, PC, MI, and DI were removed (Figure 3-1). Sections of the intestine were opened and washed with Cortland saline (7.24 g NaCl, 0.22 g $CaCl_2 \cdot 2\ H_2O$, 0.38 g KCl, 0.41 g NaH_2PO_4, 0.12 g $MgSO_4$, 1.00 g $NaHCO_3$ per L H_2O). Each tissue was frozen in liquid nitrogen and stored at -76°C.

Microsomal fraction preparations

Microsomes were prepared from individual liver, PC, MI, and DI tissues following the modified procedures of Shields and Eales (1986) (Figure 3-2). Tissues were partially thawed and minced in 4 vol (w/v) 0.1 M Tris-HCl buffer (12.12 g TRIS, 3.72 g EDTA, 1.0 M HCl per L H_2O) containing 0.25 M sucrose and 1 M Na-EDTA at pH 7.2. In a Wheaton homogenization chamber, the tissue was disrupted with a Polytron homogenizer and further broken down with 3 strokes of a motorized pestle (Tri-R Instruments, Inc.). The resulting homogenate was filtered through 2 layers of cheesecloth and transferred to 26.3-mL Beckman centrifuge tubes.

Samples were centrifuged for 20 min at 730 g to pellet the nuclear fraction using a Beckman L8-55 ultracentrifuge. The post-nuclear fraction (supernatant) was centrifuged for 20 min at 25,200 g to pellet the mitochondrial/lysosomal fraction. The supernatant containing the post-mitochondrial fraction was centrifuged for 67 min at 110,000 g to separate the microsomal pellet from the cytosol. The pellet was resuspended in 1-2 mL of buffer to obtain a protein concentration between 4-16 mg/mL. All microsomes were stored at -76°C until analysis.

TH metabolism assay

The TH metabolism assay was based on the microsomal TH deiodinase assay of Shields and Eales (1986) as adapted by Frith and Eales (1996) (Figure 3-3). Microsomal fractions were partially thawed on ice and diluted with 0.1 M Tris-HCl buffer (pH 7.2; this varied when determining assay pH conditions), containing 1 mM Na-EDTA and at 0 mM or 10 mM dithiolthreitol (DTT; this concentration varied when determining assay DTT concentration), to a final protein concentration of 0.2-0.8 mg/mL. A volume of 0.5 mL of the above preparations was pre-incubated in darkness for 30 min in a shaking water bath (12°C, 140 rpm).

After pre-incubation for 30 min in the dark, the assay reaction was initiated by adding 10 µL of either T_4 substrate or T_3 substrate to the microsomal fraction for 45 min (this varied when determining assay incubation time). For all assays, TH substrate consisted of an equal volume of non-radioactive TH added to purified radioactive TH (*TH) to produce a final substrate concentration of 0.16 nM T_4 or 0.19 nM T_3 at 10 x 10^6 cpm/mL. The TH portion of the substrate consisted of T_4 or T_3 (Sigma) dissolved in 5 µL MeOH, then brought up to final concentration in 0.1 N NaOH. The *TH portion of the substrate consisted of ^{125}I outer-ring labeled T_4 (*T_4; specific activity 1250 µCi/µg, New England Nuclear) or ^{125}I outer-ring labeled T_3 (*T_3; specific activity 3300 µCi/µg, New England Nuclear) purified on an LH-20 column, removing the free iodide contamination (I^-) with 3 mL H_2O and eluting the *TH fraction with 2.5 mL ethanolic-ammonia (EtOH:NH_4OH; 99:1 v/v). The purified *TH (>92% TH) was dried at 30°C under a stream of air and reconsituted to final concentration in 0.1 N NaOH.

For T_4 complete ORD (T_4cORD) measurement, outer-ring double radioiodine-labeled T_4 (**T_4) and single radioiodine-labeled T_4 (*T_4) were used as substrates to determine if T_4cORD activity involved the complete removal of both outer-ring I^- from the T_4 substrate.

After 30 min, the incubate reaction was stopped with 10 μL of 2 mM potassium iodide (KI). This time was varied when determining assay incubation time.

Overview of the analyses of TH metabolites and TH pathways

The incubate was separated into two aliquots (Figure 3-3). One aliquot was used to determine both total TH metabolism by using LH-20 column separation (see Total TH metabolism section) and iodoprotein (IP) production by using trichloroacetic acid (TCA) protein precipitation (see IP production section). The other aliquot was used to measure activities of specific TH metabolism pathways by using HPLC separation of radiolabeled products (see Specific TH metabolism pathways section).

Initially during determination of assay procedures, only total TH metabolism pathways and IP production were measured. However, there were limitations of the assay due to the extent of various ORD and IRD pathways active in the intestine. By using LH-20 column chromatography, only the *I^- and/or *IP released during metabolism, not the iodothyronines formed, could be measured. Increases or decreases in individual pathways could not be determined, only changes in overall amounts of TH metabolism. A more specific characterization of types of active TH metabolism pathways was undertaken by using HPLC separation of radiolabeled products. Thus, changes in activity within the pathways could be measured under altered assay conditions. Despite the greater

specificity of HPLC separation, LH-20 column separation followed by TCA protein precipitation was still necessary for measurement of IP production.

Total TH metabolism

A 200-μL aliquot of the incubate was added to an LH-20 column and eluted with 300 μL of H_2O. The eluate contained $*I^-$ removed by ORD and/or radioiodine-labeled IP (*IP) produced. However, it did not contain the iodothyronines, which remained on the column. The initial water fraction was counted for 10 min in the gamma counter (Beckman Gamma 8000 or Packard Cobra II Auto-gamma), and the radioactivity, measured, in counts per minute (cpm), was used to calculate pmoles of TH substrate converted to I^- or IP per hour per milligram of microsomal protein (Total TH metabolism; see Appendix I and II for calculations).

IP production

The total TH metabolism fraction was further analyzed to determine the proportion of its radioactivity that was *IP. For this purpose, the radioiodine-incorporated proteins were precipitated from the H_2O fraction by adding 25% TCA and centrifuging at 1420 g for 15 min in an IEC Centra-M centrifuge. After discarding the supernatant, the radioactive precipitate was counted in the gamma counter to determine *IP production. Activity was expressed as pmoles of TH substrate converted to IP per hour per milligram of microsomal protein (see Appendix III for calculations).

Specific TH metabolism pathways

To the remaining 300 μL of incubate, 650 μL of methylmercaptoimidazole (MMI) in methanol (115 mg MMI/mL methanol) was added and centrifuged at 1420 g for 5 min. A volume of 400 μL of the supernatant was analyzed in a Gilson-IBM binary gradient

HPLC with a C-18 150 x 4.6 mm Econosphere column (5 µm, Alltech) to separate *TH metabolism products using the procedures of Sweeting and Eales (1992). Products were eluted at 1 mL/min with an isocratic flow of 40% acetonitrile (0.1% trifluoroacetic acid) and 60% H_2O (0.1% trifluoroacetic acid) over 20 min.

With T_4 substrate, T_4cORD (*T_4 completely deiodinated from both positions on the outer ring so that only *I^- remained as a labeled product), T_4ORD (*T_4 deiodinated from one position on the outer ring, producing equal amounts of *T_3 and *I^-), and T_4IRD (*T_4 deiodinated from one position on the inner ring, producing only *rT_3 as a labeled product) can be followed. With T_3 substrate, T_3ORD (*T_3 deiodinated from the one remaining labeled position on the outer ring so that only *I^- remains as a labeled product) and T_3IRD (*T_3 deiodinated from one position on the inner ring, producing only *T_2) can be followed (Figure 2-1). The area under the product curve can be used to calculate the percent of TH substrate degraded to that product (Figure 3-4). Using this percent conversion and the substrate level in the incubate, activities of specific TH metabolism pathways were expressed as pmoles of TH substrate converted to product per hour per milligram of microsomal protein (see Appendix IV and V for calculations).

For T_4cORD, the amount of activity was calculated by subtracting T_4ORD (using the area under the *T_3 curve; which is equivalent to the outer-ring *I^- removed to form *T_3) from total *I^- production (using the total area under the *I^- curve). This left only the I^- removed by T_4cORD, which left no other radiolabeled products to follow. In order to obtain the HPLC data used in the calculations, proteins were precipitated with MeOH. Therefore, total I^- production did not include IP production, as seen in total T_4

metabolism calculated from the LH-20 H_2O elution (see Appendix VI for total $^*I^-$ production and T_4cORD calculations).

Assay conditions for intestinal TH metabolism

Assay conditions for PC, MI, and DI were determined by varying cofactor concentration, incubation time, and pH. In most instances, the liver TH metabolism assay was used as a reference, since optimal assay conditions were previously determined for rainbow trout deiodination pathways in hepatic tissue (Eales *et al.*, 1993).

Cofactor concentration

Cofactor requirements were determined for TH metabolism assays. The thiol cofactor allows the deiodinase enzyme to function by reducing the deiodinase enzyme, rendering the catalytic site functional (Kohrle, 1996). For microsomal assays, DTT replaces a presumed endogenous thiol cofactor, and the concentration was optimized. Glutathione (GSH), a potential endogenous thiol cofactor, was also tested in TH metabolism assays. Initially, total T_4 metabolism was measured for liver, PC, MI, and DI using ranges of thiol cofactor concentrations of 0 mM to 20 mM DTT and 0 mM to 4 mM GSH. Total T_3 metabolism was measured using 0 mM and 10 mM DTT. Next, specific TH metabolism pathways were measured by using either T_4 or T_3 substrate at both 0 mM and 10 mM DTT.

Incubation time

Incubation time for T_4 metabolism assays were tested over a range of 30 to 120 min for 0 mM DTT. These assays determined incubation time for liver, PC, MI, and DI by measuring the amount of total T_4 metabolism.

pH

Assay pH for both T_4 metabolism and T_3 metabolism was determined. For liver, PC, MI, and DI without DTT, total T_4 metabolism was examined over pH values of 6.5 to 9.5. Activities of specific T_4 metabolism pathways and T_4IP production were measured over pH values of 7.2 to 9.4. For liver and DI with 10 mM DTT, total T_4 metabolism, specific T_4 metabolism pathways, and T_4IP production were further investigated over pH values of 7.2 to 8.8. Specific T_3 metabolites and T_3IP production were measured for liver, PC, MI, and DI at 0 mM DTT and 10 mM DTT over pH values of 7.2 to 8.8.

Mechanisms of IP formation

To gain further insight into the formation of *IP, microsomes were incubated with *I$^-$ and compared with microsomes incubated with *T_4. To determine the possible source of the protein bound as IP, *T_4 substrate was incubated with 0.20 mg/mL bovine serum albumin (BSA) as a protein source instead of microsomal protein while using 0 mM or 20 mM DTT.

Statistics

Using the SPSS statistics package, the univariate analysis of variance (ANOVA) was used to compare the effects of DTT concentrations on the rate of either total T_4 metabolism or total T_3 metabolism. These were followed by LSD post hoc multiple comparisons to determine significant differences due to DTT concentrations. The tests were performed individually for liver, PC, MI, and DI. Univariate ANOVA was also used to compare the rates of each specific T_4 metabolism pathway at either 0 mM or 10 mM DTT and to compare the rates of each specific T_3 metabolism pathway at either 0 mM or 10 mM DTT. These were followed by LSD post hoc multiple comparisons to

determine significant differences among activities in the pathways. Each test was performed individually for liver, PC, MI, and DI. Results were determined statistically significant at $p \leq 0.05$. Sample sizes were too small for the other treatments to perform statistics.

Results

Effects of DTT cofactor concentration on total T_4 metabolism

DTT concentrations of 0, 5, 10, 15, or 20 mM were used to determine assay conditions for total T_4 metabolism (Figure 3-5). For the liver, total T_4 metabolism was significantly higher at 10 mM DTT (0.49 pmol T_4 converted/hr/mg protein) than at 0 mM DTT (0.16 pmol T_4 converted/hr/mg protein; $F=13.170$, d.f.=1, $p=0.001$).

For all intestinal regions (PC, MI, or DI), the highest levels of total T_4 metabolism were measured at 0 mM DTT (0.14 pmol T_4 converted/hr/mg protein for PC, 0.11 pmol T_4 converted/hr/mg protein for MI, and 0.20 pmol T_4 converted/hr/mg protein for DI). For the MI, total T_4 metabolism was significantly higher at 0 mM DTT than at 20 mM DTT ($F=11.615$, d.f.=4; $p=0.036$). It was unknown, at this point, if the same T_4 metabolism pathways were active at different levels of cofactor. Thus, further assays included cofactor concentrations of both 0 mM and 10 mM DTT. In addition, specific T_4 metabolism pathways and T_4IP production were measured for liver and intestinal regions.

Effects of DTT cofactor concentration on specific T_4 metabolism pathways and T_4IP production

DTT concentrations of 0 mM and 10 mM were used to measure specific T_4 metabolism pathways (T_4cORD, T_4ORD, and T_4IRD) and T_4IP production (Figure 3-6).

In the liver, T_4ORD at 0 mM DTT was significantly greater than the other specific T_4 metabolism pathways (F=7.542, d.f.=3, p=0.001).

For all regions of the intestine, T_4cORD did not require DTT and was significantly greater than other specific T_4 metabolism pathways (F=5.307, d.f.=3, p=0.004 for PC; F=1.912, d.f.=3, p=0.035 for MI; F=10.533, d.f.=3, p=0.000 for DI). The PC (0.40 pmol T_4 converted/hr/mg protein) and the DI (0.47 pmol T_4 converted/hr/mg protein) were more active than the MI (0.15 pmol T_4 converted/hr/mg protein).

For all intestinal regions, DTT decreased T_4cORD activity but increased T_4ORD activity so that T_4cORD was no longer significantly greater than the other specific T_4 metabolism pathways (F=0.654, d.f.=3, p=0.632 for PC; F=3.056, d.f.=3, p=0.122 for MI; F=2.691, d.f.=3, p=0.109 for DI). Thus, T_4ORD functions optimally with cofactor in both liver and intestine.

Effects of DTT cofactor concentration on total T_3 metabolism

Total T_3 metabolism was measured for liver, PC, and DI at 0 mM and 10 mM DTT (Figure 3-7). Liver total T_3 metabolism was low, with little difference between 0 mM and 10 mM DTT (0.04 vs. 0.03 pmol T_3 converted/hr/mg protein).

For intestinal assays, total T_3 metabolism varied with intestinal region and DTT concentration. Only PC total T_3 metabolism was significantly greater at 10 mM DTT than at 0 mM DTT (F=6.324, d.f.=1, p=0.036). However, DI total T_3 metabolism was greater, but not significantly (F=4.090, d.f.=1, p=0.078), without cofactor than with cofactor. Total T_3 metabolism in PC and DI exceeded that of the liver.

Effects of DTT cofactor concentration on specific T_3 metabolism pathways and T_3IP production

T_3ORD and T_3IRD pathways and T_3IP production were measured with or without DTT (Figure 3-8). All T_3 metabolism pathways were low in liver, with T_3IRD, which produces T_2 from T_3, being the most prominent pathway at 10 mM DTT (0.008 pmol T_3 converted/hr/mg protein).

As seen when measuring total T_3 metabolism in intestinal assays, activities of specific TH metabolism pathways varied with intestinal region. Overall, no specific T_3 metabolism pathways were significantly greater than the other for all intestinal regions. For PC, T_3IRD activity was greatest (0.05 pmol T_3 converted/hr/mg protein). For MI, T_3ORD activity was greatest (0.05 pmol T_3 converted/hr/mg protein. For DI, both T_3ORD and T_3IRD were active without cofactor (0.11 pmol T_3 converted/hr/mg protein and 0.06 pmol T_3 converted/hr/mg protein, respectively). For all intestinal regions, T_3IP production was consistently low (range: 0.0017-0.0018 pmol T_3 converted/hr/mg protein). Again, DI was more active at metabolizing T_3 than liver or other intestinal regions, and these pathways did not require cofactor.

Effects of GSH cofactor concentration on total T_4 metabolism

Since DTT was not required for T_4cORD, the prominent T_4 metabolism pathway in the intestine, another possible cofactor, GSH, was tested in assays measuring total T_4 metabolism (Figure 3-9). In the liver, total T_4 metabolism was not altered with increases in GSH cofactor concentration.

In the presence of GSH, the total T_4 metabolism in intestinal regions was depressed markedly. Without GSH as a cofactor, PC exhibited the greatest metabolism

(0.62 pmol T_4 converted/hr/mg protein), followed by DI (0.19 pmol T_4 converted/hr/mg protein), and then MI (0.14 pmol T_4 converted/hr/mg protein). Since GSH did not increase total T_4 metabolism, it was not tested further, and both 0 mM and 10 mM DTT were routinely used in subsequent assays.

Effects of incubation time on total T_4 metabolism

The effects of incubation time on total T_4 metabolism (minus the T_4IP products) were tested for liver, PC, MI, and DI without DTT (Figure 3-10). Maximal total T_4 metabolism was reached in liver by 30 min.

Similar results showing maximal total T_4 metabolism at 30 min were found for the intestinal regions. Again, the distal region exhibited the greatest amount of total T_4 metabolism with 0.61 pmol T_4 converted/hr/mg protein. Thus, a 45-min incubation time was routinely used to allow maximal TH metabolism to occur and to allow time for an adequate number of samples and replicates to be assayed.

Effects of pH on T_4 metabolism pathways without DTT

The effects of pH on T_4 metabolism pathways without DTT were tested for liver, PC, MI, and DI. When measuring total T_4 metabolism, a pH range of 6.5 to 9.5 (specifically 6.5, 7.0, 7.25, 7.5, 8.0, 8.75, and 9.5) was used (Figure 3-11). Specific T_4 metabolism pathways and T_4IP production were determined over pH values of 7.2, 8.0, 8.8, and 9.4 (Figures 3-12 through 3-15).

For liver, total T_4 metabolism did not differ greatly with changes in assay pH, and activity was highest at pH 8.0 (0.06 pmol T_4 converted/hr/mg protein). Higher total T_4 metabolism at pH 8.0 was due to increased T_4cORD (0.08 pmol T_4 converted/hr/mg protein), T_4ORD (0.06 pmol T_4 converted/hr/mg protein), and T_4IRD (0.02 pmol T_4

converted/hr/mg protein) (Figure 3-12). However, T_4IP formation was low, with optimal pH at 8.8 (0.006 pmol T_4 converted/hr/mg protein).

All intestinal regions showed greater total T_4 metabolism with increased pH (Figure 3-11). For example, PC total T_4 metabolism was similar to liver until pH 8.8. PC at higher pH (8.75 and 9.5) showed increased metabolism (0.22 and 0.21 pmol T_4 converted/hr/mg protein, respectively). A similar trend was observed for MI and DI with increased total T_4 metabolism at higher pH.

To investigate the increased total T_4 metabolism at higher pH, specific T_4 metabolism pathways and T_4IP production were determined at a pH range of 7.2 to 9.4 for all intestinal regions. For PC, pH 8.0 was optimal for T_4cORD (0.06 pmol T_4 converted/hr/mg protein) (Figure 3-13). T_4ORD activity was highest at pH 9.4 (0.03 pmol T_4 converted/hr/mg protein). T_4IRD activity was highest at pH 8.8 (0.025 pmol T_4 converted/hr/mg protein). T_4IP formation increased with increasing pH, with the highest levels at pH 8.8 and 9.4. Therefore, the increased total T_4 metabolism with increased pH seen in LH-20 column separation was attributed to increased T_4cORD and T_4IP production.

For MI, pH 8.8 was optimal for T_4cORD (0.004 pmol T_4 converted/hr/mg protein), with no activity detectable at lower pH (Figure 3-14). T_4ORD activity was highest at both pH 7.2 and pH 9.4 (0.02 pmol T_4 converted/hr/mg protein for both). T_4IRD activity was highest at pH 8.8 (0.02 pmol T_4 converted/hr/mg protein). T_4IP formation increased with increasing pH, and pH 9.4 produced the greatest T_4IP (0.04 pmol T_4 converted/hr/mg protein). Besides T_4IP production, almost all T_4 metabolism pathways in MI were active at higher pH.

For DI, pH 7.2 was the optimal pH for T_4cORD (0.48 pmol T_4 converted/hr/mg protein), with negligible activity detectable at other pH values (Figure 3-15). T_4ORD activity was highest at pH 7.2 (0.04 pmol T_4 converted/hr/mg protein), but it was consistently low at other pH. T_4IRD activity was also consistently low at all pH except pH 9.4, where no rT_3 was detected. T_4IP production was also low, however, increasing the pH increased IP formation to 0.07 pmol T_4 converted/hr/mg protein. Thus, pH 7.2 was the optimal assay pH for DI, one of the most active regions of the intestine for T_4 metabolism, and T_4IP was the main active pathway at higher pH.

Effects of pH on T_4 metabolism pathways with DTT

The assay pH was also determined for total T_4 metabolism with 10 mM DTT by focusing on the liver and the most active intestinal region, DI at pH 7.2, 8.0, and 8.8 (Figure 3-16). Specific T_4 metabolism pathways were measured at pH 7.2 and pH 8.0 (Figures 3-17 and 3-18).

For liver, total T_4 metabolism was highest at pH 7.2 (Figure 3-16). The higher T_4 metabolism at pH 7.2 was due to high T_4ORD activity (Figure 3-17).

For DI, total T_4 metabolism activity was similar at all pH values, with activity ranging from 0.14 to 0.15 pmol T_4 converted/hr/mg protein (Figure 3-16). The most active pathway was T_4IRD at pH 7.2 (0.05 pmol T_4 converted/hr/mg protein) (Figure 3-18). At this pH, T_4ORD and T_4IP were also active (0.03 pmol T_4 converted/hr/mg protein and 0.01 pmol T_4 converted/hr/mg protein, respectively). As pH increased to 8.8, T_4IRD was non-detectable, and T_4ORD increased. T_4IP production was unaltered by pH. Thus, even though total T_4 metabolism did not change with increased pH, measuring specific T_4

metabolism indicated a decrease in pathways inactivating T_4 and an increase in pathways activating T_4.

Effects of pH on T_3 metabolism pathways without DTT

To determine assay pH for T_3 metabolism without DTT, specific T_3 metabolism pathways were measured for liver, PC, MI, and DI at pH 7.2, 8.0, and 8.8.

For liver, T_3ORD activity increased from 0 to 0.005 pmol T_3 converted/hr/mg protein with increased pH (Figure 3-19). T_3IRD activity was active at pH 8.0 only (0.008 pmol T_3 converted/hr/mg protein). T_3IP production was present at pH 7.2 only (0.005 pmol T_3 converted/hr/mg protein).

For PC, increasing pH increased T_3ORD activity only slightly to levels above non-detectable (Figure 3-20). T_3IRD activity was highest at pH 7.2, decreasing with increased pH (0.05 to 0.003 pmol T_3 converted/hr/mg protein). T_3IP production was similar at all pH concentrations (0.01 pmol T_3 converted/hr/mg protein).

For MI, T_3ORD was the most active pathway, increasing with increased pH (0.05 pmol T_3 converted/hr/mg protein to 0.09 pmol T_3 converted/hr/mg protein) (Figure 3-21). T_3IP production was minimally active and only at pH 8.8 (0.002 pmol T_3 converted/hr/mg protein).

For DI, T_3ORD was the most active pathway, with highest levels at pH 7.2 (0.12 pmol T_3 converted/hr/mg protein) (Figure 3-22). T_3IRD was also active at pH 7.2 (0.08 pmol T_3 converted/hr/mg protein), decreasing to non-detectable at pH 8.0 and 0.02 pmol T_3 converted/hr/mg protein at pH 8.8. T_3IP production was low at all pH values. Overall, DI was the most active intestinal region for T_3 metabolism, even surpassing liver activity; pH 7.2 was optimal for the assay.

Effects of pH on T_3 metabolism pathways with DTT

To determine optimal assay pH for T_3 metabolism with DTT, specific T_3 metabolism pathways were measured for liver, PC, MI, and DI at pH 7.2, 8.0, and 8.8.

For liver, T_3IRD was the most active pathway with the highest activity at pH 8.0 (0.04 pmol T_3 converted/hr/mg protein) (Figure 3-23). T_3IP production fluctuated little with pH (range of 0.01 to 0.02 pmol T_3 converted/hr/mg protein).

For PC, T_3IRD was the most active pathway, but it was only detectable at pH 7.2 (0.02 pmol T_3 converted/hr/mg protein) (Figure 3-24). T_3IP production was consistently active (0.02 pmol T_3 converted/hr/mg protein), thus it was insensitive to pH over the range 7.2-8.0.

DI T_3 metabolism was similar to that for PC (Figure 3-25). T_3IRD activity was highest at pH 7.2 (0.07 pmol T_3 converted/hr/mg protein), decreasing to non-detectable levels at pH 8.8. T_3IP production did not change with a change in pH (0.03 pmol T_3 converted/hr/mg protein). Again, DI was the most active intestinal region for T_3 metabolism with DTT; pH 7.2 was optimal for the assay. T_3IRD, whether in liver or intestine, functions optimally with DTT.

Mechanism of IP formation

Since the pathway for IP production and the actual products formed were unknown, either $*T_4$ or $*I^-$ substrates were incubated with intestinal microsomes. No IP production occurred with $*I^-$ as a substrate (data not shown). Therefore, the production of IP depended upon TH as a substrate, not the $*I^-$ released from metabolism.

$*T_4$ substrate was incubated with 0.2 mg/mL of BSA as a protein source, instead of microsomal protein, using 0 mM or 20 mM DTT (Figure 3-26). IP production

occurred with BSA, and so was formed regardless of protein source or presence of enzymes. An increase in cofactor decreased TH interaction with proteins.

Discussion

This chapter set out to describe TH metabolism pathways in specific segments of the intestine (PC, MI, and DI) of the rainbow trout, using liver as a reference standard. Using the microsomal TH metabolism assay, DTT concentration, incubation time, and pH requirements were characterized for the active pathways measured.

Previous studies on the trout liver have optimized conditions for deiodination assays. In the present study, T_4ORD was the predominant hepatic deiodination pathway that deiodinated T_4 to T_3, and assay conditions were established as 10 mM DTT, 45 min incubation time, and pH 7.2. Substrate concentrations of 0.16 nM T_4 or 0.19 nM T_3 were used to simulate likely tissue TH levels. This supports previous literature both with regard to optimal T_4ORD assay conditions and with regard to the liver's role as a main regulator of peripheral TH metabolism and in maintaining T_3 homeostasis (Eales *et al.*, 1993; Eales, 1995).

Intestinal TH metabolism pathways were more complex than those in the liver, and intestinal TH metabolism had different assay requirements than that of the liver. Of all of the intestinal regions studied for TH metabolism, the DI was the most active. The DI did not require cofactor for the most active pathway, T_4cORD. Hence, assays were run at both 0 mM and 10 mM DTT. Other than cofactor requirements, assay conditions were kept similar to the hepatic TH metabolism assay. Substrate concentrations of 0.16 nM T_4 or 0.19 nM T_3 were used at pH 7.2 with 45 min incubation time.

Besides not requiring cofactor, overall levels of intestinal TH metabolism activity were lower with T_4 substrate but higher with T_3 substrate than in the liver. However, pathways that were low in the liver were more active and predominated in the intestine. Intestinal deiodination in the Atlantic cod (Cyr *et al.*, 1997) and American plaice (Adams *et al.*, 2000) were also low under normal physiological conditions. In sturgeon, intestinal TH metabolism was significant, but it was lower than hepatic deiodination (Plohman *et al.*, 2002). However, in lampreys (Eales *et al.*, 1997, 1999) and hagfish (McLeese *et al.*, 2000), intestinal deiodination (T_4ORD) predominated over hepatic deiodination. Thus, the intestinal deiodination may represent ancestral TH metabolism pathways that are actively retained in more "primitive" fish lineages but still present in teleost fish.

In summary, in trout under normal physiological conditions, while the liver mainly deiodinated T_4 to form T_3, the intestine showed a broader array of deiodination and TH metabolism pathways geared toward degradation of T_4 and in particular degradation of T_3. Thus, the main ways in which the intestine differed from the liver were that T_4cORD, T_4IRD, and TH IP production was active, and no DTT was required as a cofactor. These unusual properties of intestinal TH metabolism are discussed below.

T_4cORD

Complete outer-ring deiodination has not been characterized in other tissues for any species. For this TH metabolism pathway, all of the $^*I^-$ was removed without forming other iodothyronines. Thus, this is considered an inactivating pathway. T_4cORD was, at the very least, removing all of the outer-ring I^-. This was shown by using double-labeled T_4. $^*I^-$ production from T_4cORD doubled with the double-labeled substrate. Since inner-ring I^- presence or removal could not be measured without an outer-ring

radioiodine-labeled iodothyronine to follow, it was not known if all of the I^- (both outer-ring and inner-ring) were removed.

Similar to T_4cORD, T_3ORD represents complete outer-ring deiodination. Again, it was not known if T_3ORD represented the removal of both outer-ring and inner-ring I^-. Regardless, it was similar in characteristics to T_4cORD. Both T_4cORD and T_3ORD are active without DTT, in fact, they are more active in its absence. Additionally, both are highest in the DI. Intestinal tissue may not contain an endogenous equivalent to the thiol cofactor. Thus, these degradative pathways may be prominent in the DI. They may serve to degrade excess TH potentially entering the intestine through the diet, from the circulation, or from the bile. This would prevent elevated levels of TH from entering or re-entering the circulation. Since activity was higher in the DI, it may serve to salvage I^- before loss through excretion.

IP production

IP production was further investigated, since its formation was prominent in the intestinal TH metabolism assay. IP production may have been overlooked in tissues such as the liver, since the levels of activity of other TH metabolism pathways were relatively high. IP production would be insignificant, in comparison, and it has probably been disregarded as background. TH metabolism pathways in the intestine were more numerous, however, the products of the pathways were often low in comparison to the liver. Therefore, the "background" of IP production was sufficiently conspicuous to be worthy of measurement.

The identity of the IP, much less the pathway of formation, is poorly understood. Of the TH substrates, T_4 produced more IP than T_3. However, it was not known if IP

production was dependent upon TH itself as a substrate or I^- released from the TH. A possible pathway was that $*I^-$ removed from the TH outer-ring was bound to proteins, just as I^- is bound to proteins in trout plasma (Leloup, 1970). If this was true, then it would be expected that I^- alone would serve as a substrate in the assay, resulting in I^- bound to protein. However, no *IP products were formed with $*I^-$ as a substrate. This finding was supported by Galton and Ingbar (1961) and Tata (1960) studying IP formation in rats, mice, tadpoles, and frogs. Both agreed that iodide was not the substrate for protein iodination. Thus, IP production required TH, and preferably T_4, as a substrate.

It was also not known what was the source of protein for IP production. T_4IP formation was tested using either microsomes or BSA as the protein source in the assay. IP were formed with both protein sources, indicating that TH were spontaneously, covalently bound to protein in the assay. Tata (1960) believed that proteins such as lysozyme and egg albumin could be iodinated during the deiodination of T_4. The data in this chapter do not support the need for enzymatic deiodination of T_4 to generate IP, since the incubation of T_4 with BSA did not include any source of enzyme. IP production may be a result of spontaneous degradation of TH. However, it may be linked in some way to deiodination as an increase in deiodination in the intestine appeared to increase IP production. Since DTT reduced the production of IP, the cofactor may stabilize TH, reduce spontaneous degradation of TH, and prevent IP formation in the assay. The intestine, with its lack of cofactor requirements may represent a different enzymatic environment which allows greater formation of IP.

Since the pathway for IP production and its identity are not known, it is difficult to speculate on their physiological role. Proteins in the intestinal cells may bind TH for

transport into the circulation. Binding of TH could also promote TH excretion in a manner similar to Fe^{+2} excretion in the intestine. Ferritin, an epithelial cell protein, binds excess Fe^{+2}, preventing its absorption, while the Fe^{+2} that is not bound to ferritin can be absorbed into the circulation. The Fe^{+2} that is bound to ferritin is stored intracellularly so that when the epithelial layer is sloughed off, the Fe^{+2} is excreted (Vander *et al.*, 1994). IP may serve a similar role as the protein-bound Fe^{+2}, but in this case facilitating the excretion of TH.

Dependence upon thiol cofactor DTT

In all regions of the intestine (PC, MI, and DI), 0 mM DTT produced the greatest amount of total T_4 metabolism. This suggested that enzyme characteristics for TH metabolism pathways in the intestine differed from the liver. After measuring specific T_4 products, it was shown that varying DTT concentration affected some deiodination pathways, but not others. In all regions, T_4cORD was the prominent pathway without cofactor, with DI T_4cORD activity the highest. With cofactor (10 mM DTT), T_4ORD was the prominent pathway in all regions, except the MI. At this level of cofactor, T_4ORD remained high.

Intestinal T_3 metabolism also varied with changes in cofactor concentration. In PC, 10 mM, not 0 mM DTT, produced optimal T_3 metabolism. The opposite was true in DI. Only the DI exhibited activity for both T_3ORD and T_3IRD. Both were active at 0 mM DTT, but only T_3IRD was not inhibited by DTT.

It was shown previously that thiol cofactors such as DTT, dihydrolipoamide, or monoglutathione provide an exogenous thiol that significantly increased trout microsomal deiodination activity in the liver (Pimlott and Eales, 1983; Shields and Eales, 1986).

Sulfhydryl groups or selenocysteine residues were found at the deiodinase enzyme catalytic site in mammals (Visser, 1979; Kohrle, 1996). Thiols reduced the deiodinase enzyme, forming a catalytically active enzyme. The exogenous thiol cofactor is believed to replace an endogenous biological equivalent. However, the nature of an endogenous cofactor in fish is not further understood.

Results from the present study using GSH indicated that it is not an effective cofactor for the liver or the intestine. For the liver, adding GSH did not increase activity, as was seen at 10 mM DTT. In mammals, GSH was an effective thiol cofactor for ORD in intestinal tissues (Galton *et al.*, 1991) and hepatic and kidney tissues (Goswami and Rosenberg, 1988). In fact, Galton *et al.*, (1991) suggested that GSH may be an activator of ORD *in vivo*. Yet, the present study showed that GSH was a less effective cofactor than DTT for rainbow trout liver, and it inhibited TH metabolism pathways in the intestine. Thus, GSH is not a likely endogenous cofactor for rainbow trout.

The atypical requirements for thiol cofactor in the intestine suggested a different enzyme or mechanism for intestinal TH metabolism than that found in the liver. Since the intestinal lumen itself is a reduced environment, there may be an abundance of reduced "cofactors" that can enter the intestinal cells, aiding the deiodinase enzyme in metabolism. Hence, intestinal T_4 metabolism pathways may have adapted to a different cofactor. On the other hand, the pathways may have evolved without the need for a thiol cofactor. In fact, it is not known if there is an endogenous cofactor. *In vivo*, deiodinase enzymes may not be regenerated by thiol cofactor (Kohrle *et al.*, 1993). Binding and deiodination may promote enzyme tranlocation and degradation (St. Germain and

Croteau, 1989). Therefore, the significance of *in vitro* deiodination with and without thiol cofactor is unknown.

Thus, the results confirmed that 0 mM and 10 mM DTT would be required for the microsomal assay to quantify the various TH metabolism pathways in the trout intestine. On one hand, T_4cORD and T_3ORD were inhibited by thiol cofactors, but on the other hand, T_4ORD, T_4IRD, and T_3IRD functioned at 10 mM DTT. Therefore, assays were conducted at both 0 and 10 mM DTT in order to measure pathways that required thiol cofactor as well as pathways that were inhibited by thiol cofactor.

Conclusions

TH metabolism assay conditions were defined for both the liver and the intestine. To standardize the experiments for comparisons in future experiments with these tissues, the microsomal assays were to be run under the following conditions: 0 mM and 10 mM DTT, 45 min incubation time, pH 7.2, and 0.16 nM T_4 substrate or 0.19 nM T_3 substrate.

The differences in TH metabolism between the liver and the intestine indicated different roles in the regulation of thyroid hormones. In the liver, TH metabolism pathways were activating (T_4ORD) or inactivating (T_3IRD) based on circulating T_3 levels (Eales *et al.*, 1993; Eales, 1995). Under normal, non-stressful conditions, the predominant pathway for maintaining homeostasis was T_4ORD, as was seen in this study.

In the intestine, TH metabolism pathways were deactivating. The predominant pathways were T_4cORD and IP production, leading to degradation of the TH substrate. However, many other pathways were active, such as T_4ORD, T_4IRD, T_3ORD, and T_3IRD. Together, these would deiodinate T_4 and T_3, removing a potential source of biologically

active T_3. Since intestinal TH metabolism was greatest in the DI, this region may salvage I^- before loss through the excretion.

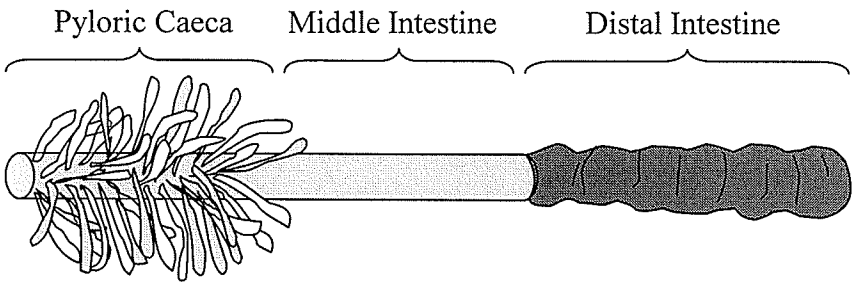


Figure 3-1. Diagram of regions of intestine sampled, including the pyloric caeca (PC), middle intestine (MI), and distal intestine (DI).

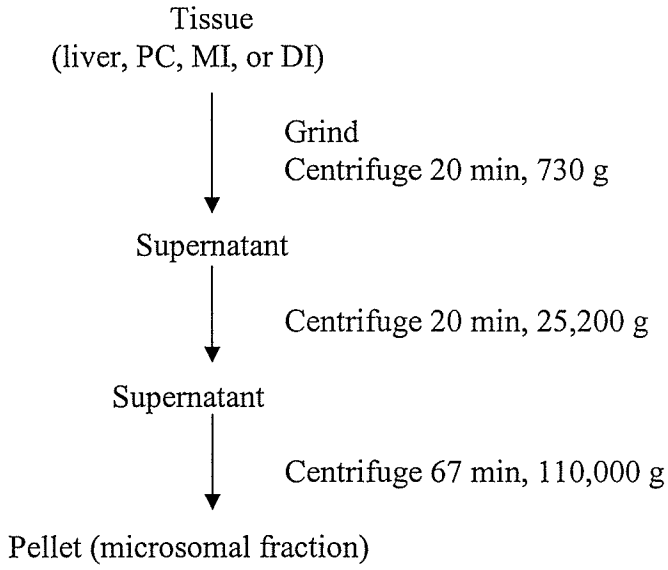
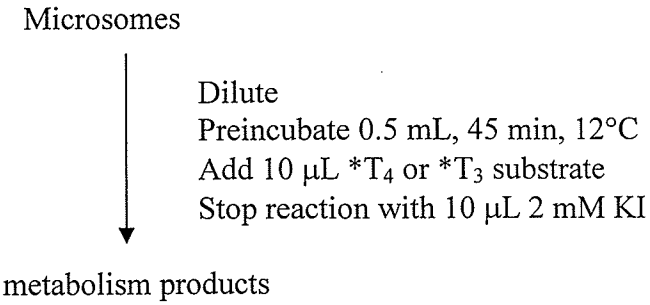


Figure 3-2. Methods for preparing microsomal fractions from liver and regions of the intestine (PC, MI, and DI) used in the *in vitro* TH metabolism assays.

TH Metabolism Assays



TH Metabolism Analysis

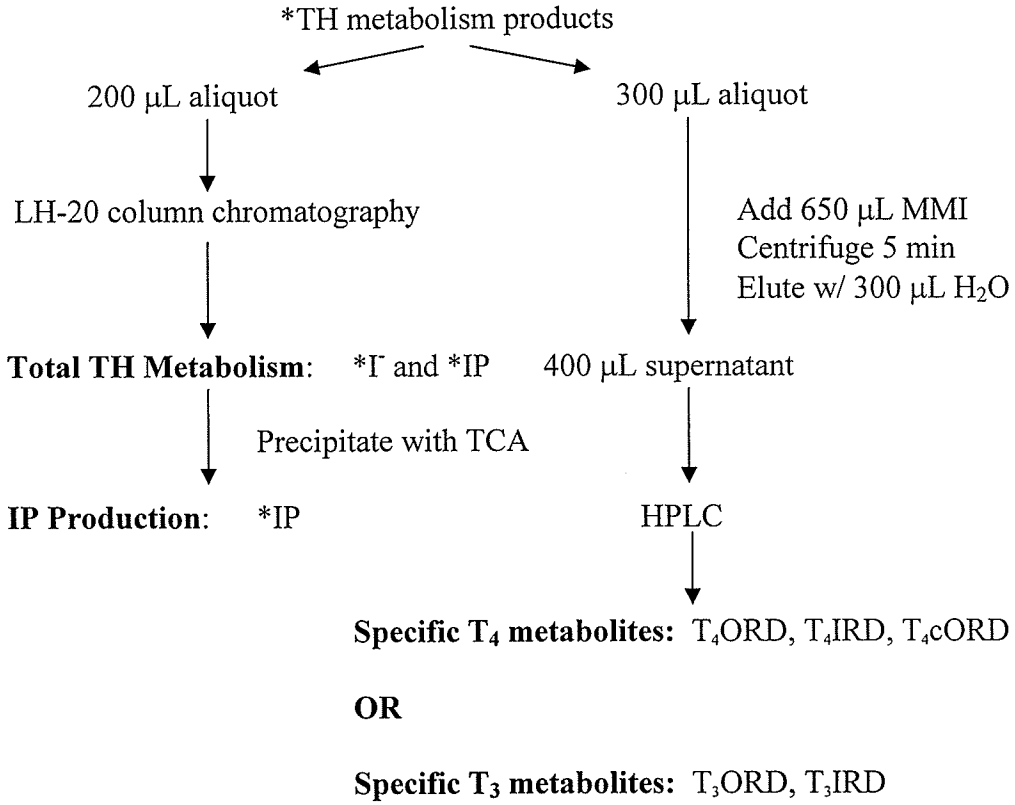
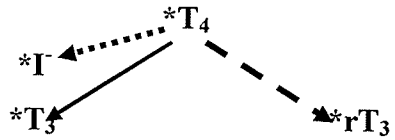


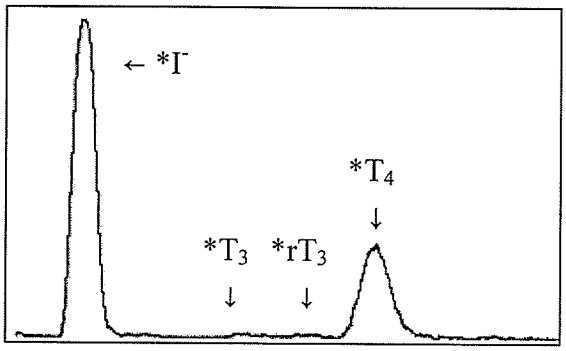
Figure 3-3. Procedures for TH metabolism assay and analysis of products formed. TH labeled with ^{125}I on the outer ring (*T_4 or *T_3) allowed measurement of the products formed from various active TH metabolism pathways. The products were analyzed using LH-20 column separation to determine total TH metabolism, followed by protein precipitation with trichloroacetic acid (TCA) to determine TH iodoprotein (IP) production. In addition, the products, after adding methylmercaptoimidazole (MMI) were analyzed using high performance liquid chromatography (HPLC) to separate specific T_4 metabolites to determine active pathways for T_4 metabolism (T_4ORD, T_4IRD, T_4cORD) or to separate specific T_3 metabolites to determine active pathways for T_3 metabolism (T_3ORD, T_3IRD).

PATHWAYS



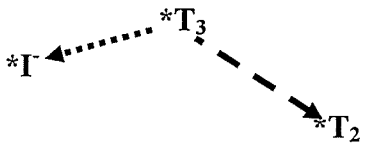
HPLC

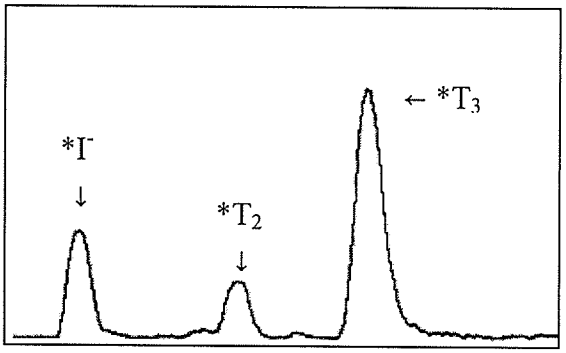
T_4 Substrate



Specific T_4 metabolites
T_4ORD: *T_3 peak
T_4IRD: *rT_3 peak
T_4cORD: *I^- peak-*T_3 peak

T_3 Substrate





Specific T_3 metabolites
T_3ORD: *I^- peak
T_3IRD: *T_2 peak

Figure 3-4. Specific TH metabolism pathways and the representative HPLC radioactivity profiles demonstrating the specific TH metabolites determined from the area under the curve.

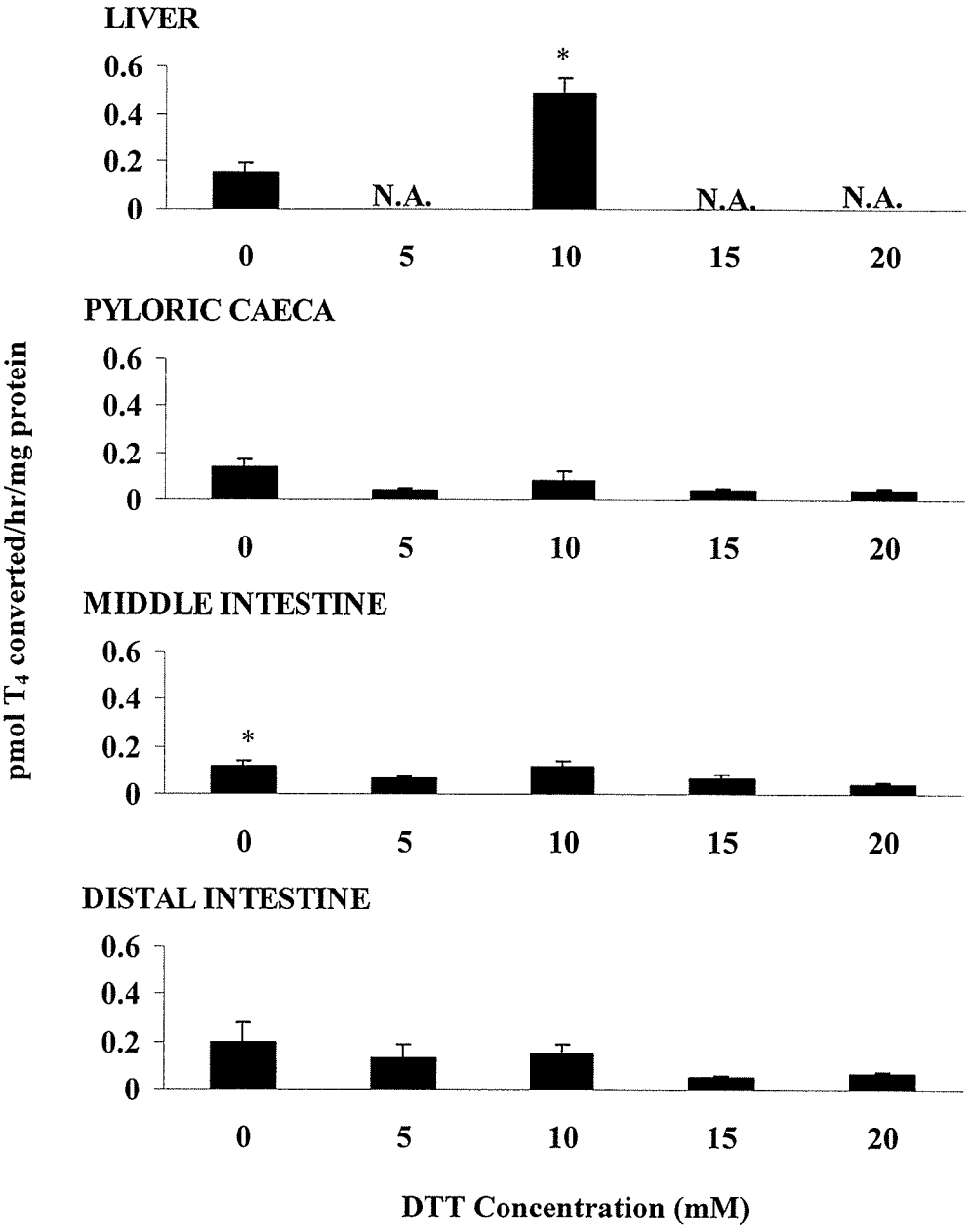


Figure 3-5. Rate of total T_4 metabolism (presumed $*I^-$ and/or *IP) at DTT concentrations of 0, 5, 10, 15, or 20 mM as determined by LH-20 column chromatography after $*T_4$ incubation with liver (n=12), pyloric caeca (n=22), middle intestine (n=14), or distal intestine (n=25) microsomes. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein (±s.e.). N.A. denotes not assayed. Asterisks indicate significant differences at $p \leq 0.05$.

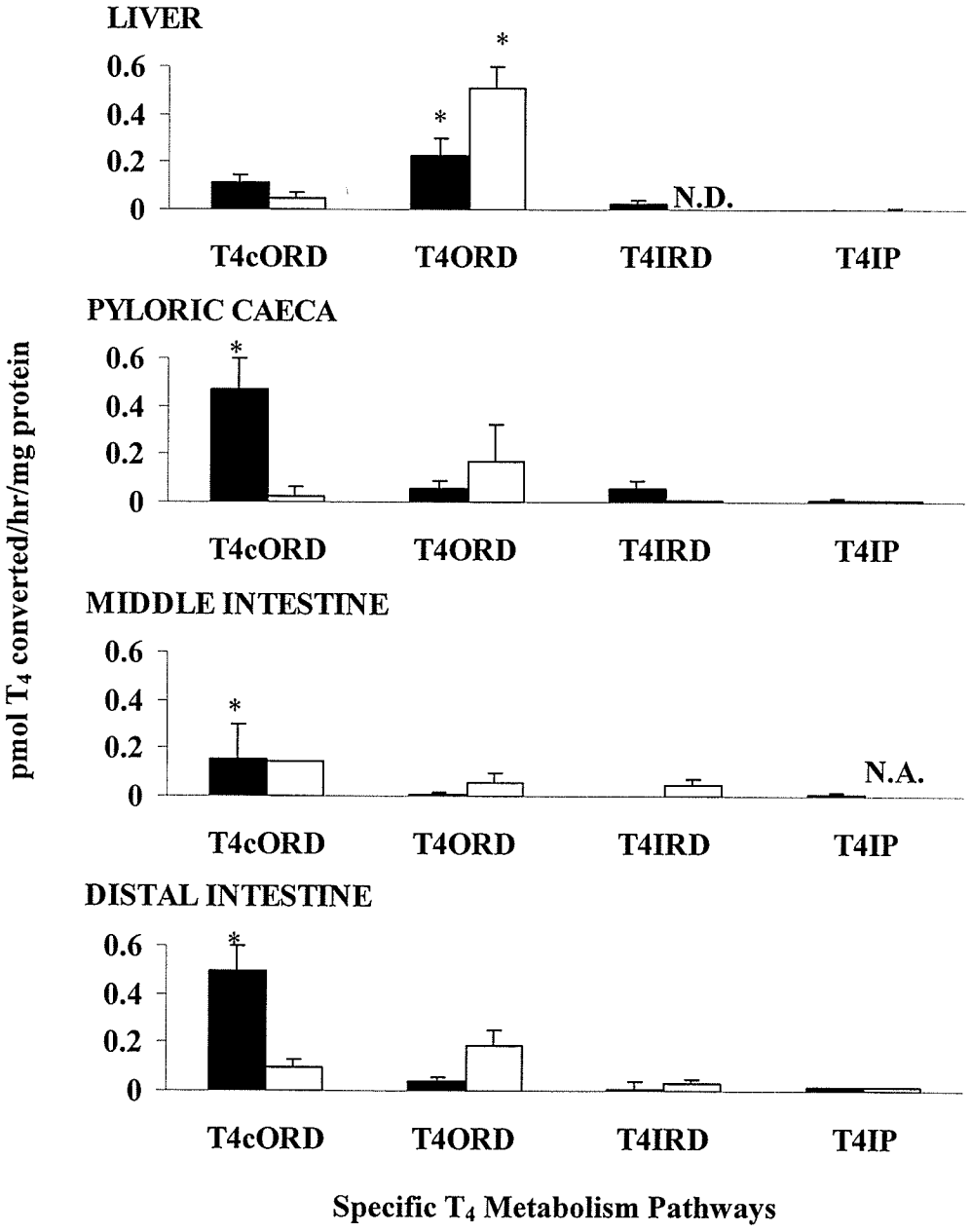


Figure 3-6. T_4cORD, T_4ORD, and T_4IRD activities at DTT concentrations of 0 mM (■) or 10 (□) mM as determined by HPLC separation of *I⁻, *T_3, and *rT_3 as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after *T_4 incubation with liver (n=6), pyloric caeca (n=10), middle intestine (n=3), or distal intestine (n=18) microsomes. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein (±s.e.). N.A. denotes not assayed. N.D. denotes non-detectable. Asterisks indicate significant differences at $p \leq 0.05$.

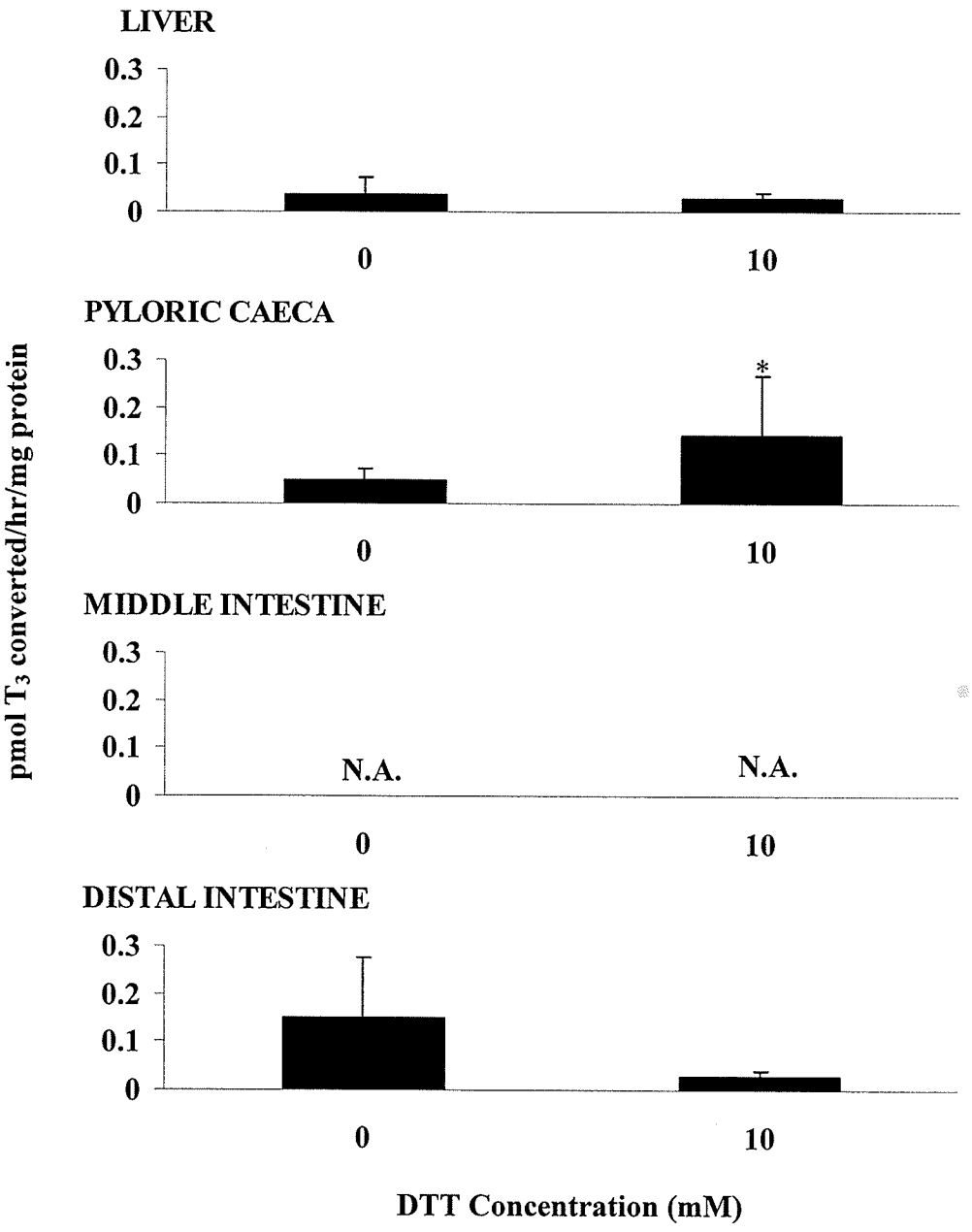


Figure 3-7. Rate of total T_3 metabolism (presumed *I$^-$ and/or *IP) at DTT concentrations of 0 or 10 mM as determined by LH-20 column chromatography after *T_3 incubation with liver (n=21), pyloric caeca (n=7), middle intestine (n=0), or distal intestine (n=4) microsomes. Activities were calculated as pmoles of T_3 converted to product per hour per milligram of microsomal protein (±s.e.). N.A. denotes not assayed. Asterisks indicate significant differences at $p \leq 0.05$

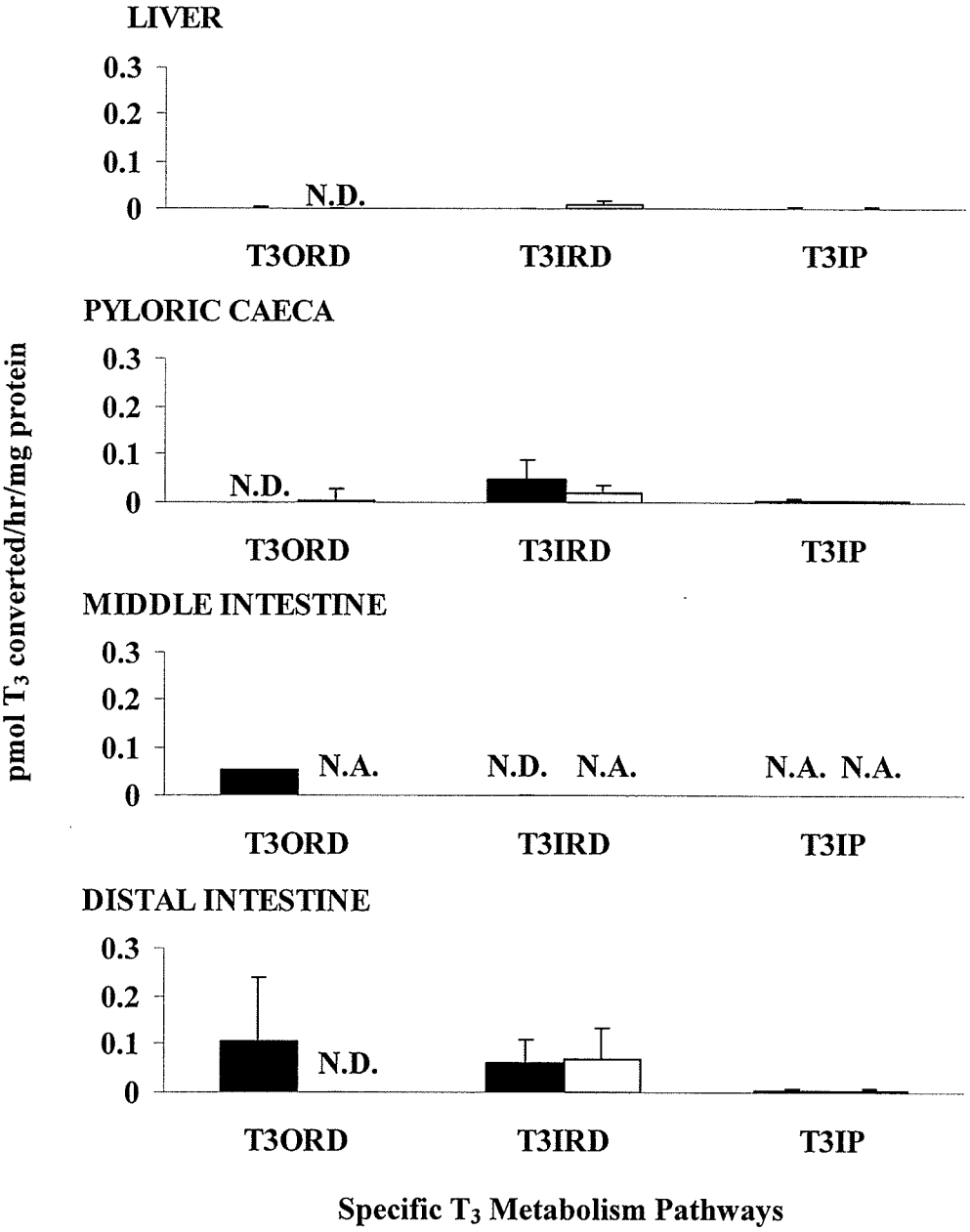


Figure 3-8. T_3ORD and T_3IRD activities at DTT concentrations of 0 (■) or 10 (□) mM as determined by HPLC separation of $*I^-$ and $*T_2$ as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_3$ incubation with liver (n=7), pyloric caeca (n=3), middle intestine (n=3), or distal intestine (n=4) microsomes. Activities were calculated as pmoles of T_3 converted to product per hour per milligram of microsomal protein (±s.e.). N.A. denotes not assayed. N.D. denotes non-detectable.

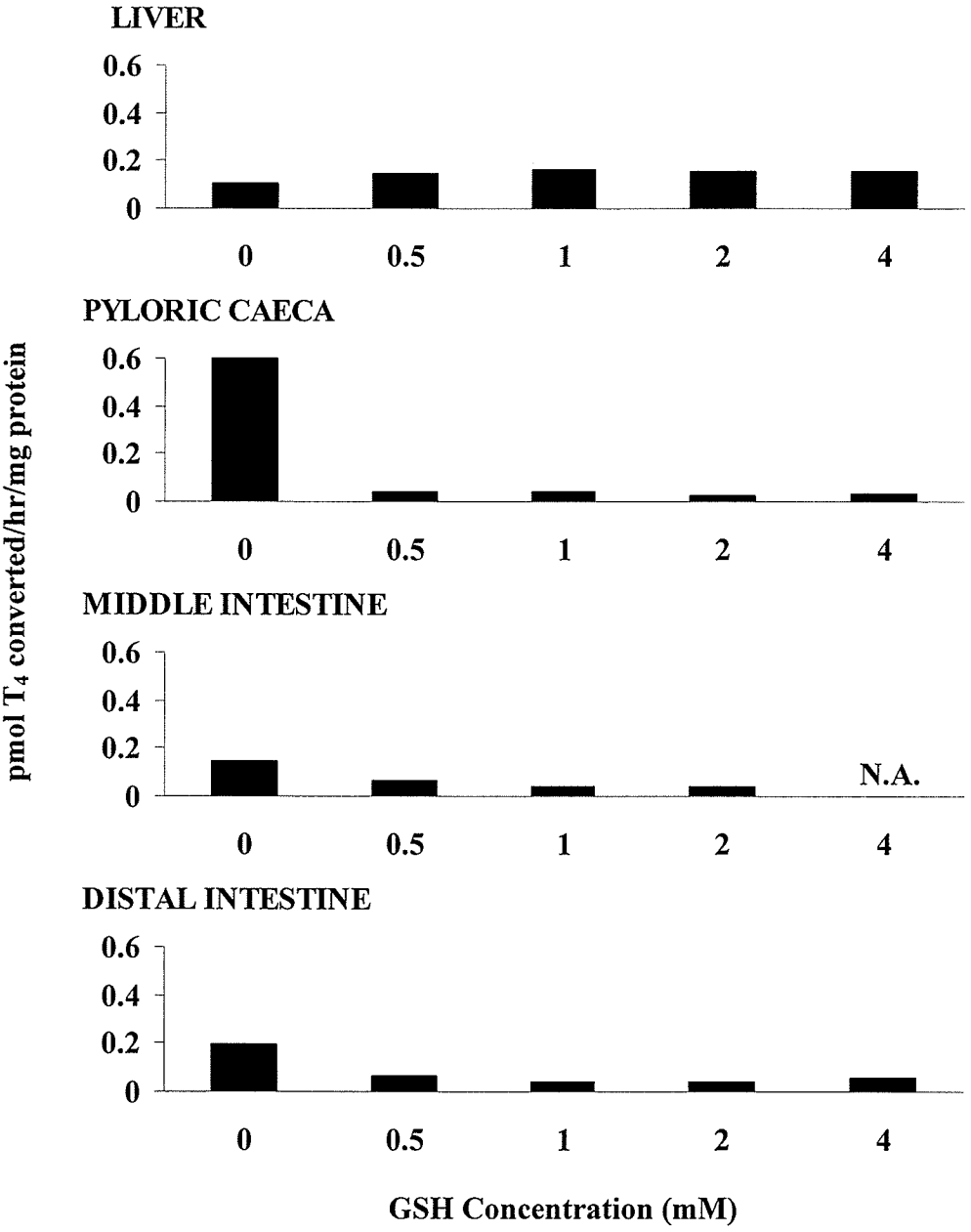


Figure 3-9. Rate of total T_4 metabolism (presumed $*I^-$ and/or *IP) at glutathione (GSH) concentrations of 0, 0.5, 1, 2, or 4 mM as determined by LH-20 column chromatography after $*T_4$ incubation with liver (n=1), pyloric caeca (n=1), middle intestine (n=1), or distal intestine (n=1) microsomes. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein. N.A. denotes not assayed.

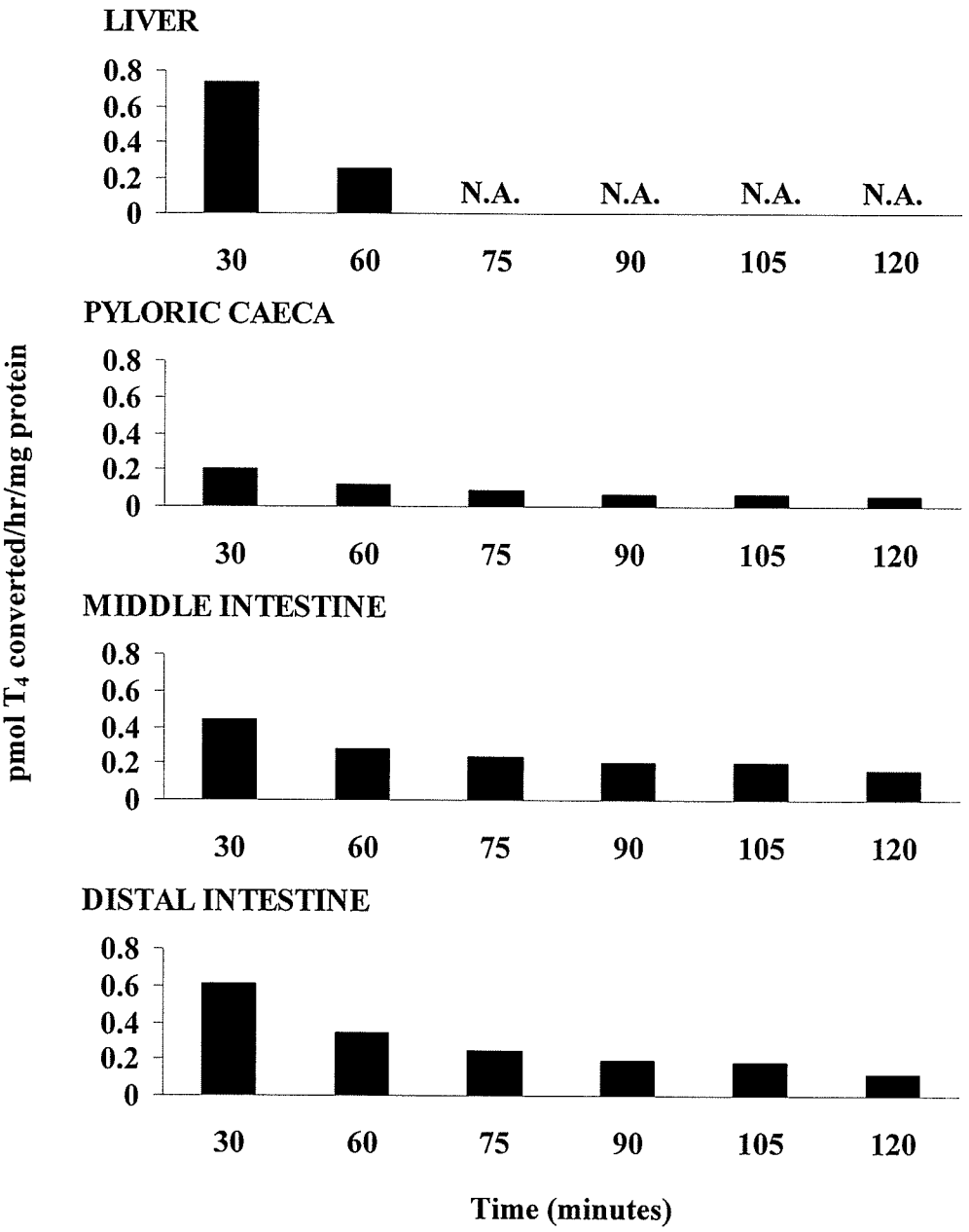


Figure 3-10. Rate of total T_4 metabolism (presumed $*I^-$ and/or *IP) without DTT over a range of incubation times (30, 60, 75, 90, 105, or 120 min) as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_4$ incubation with liver (n=1), pyloric caeca (n=1), middle intestine (n=1), or distal intestine (n=1) microsomes. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein. N.A. denotes not assayed.

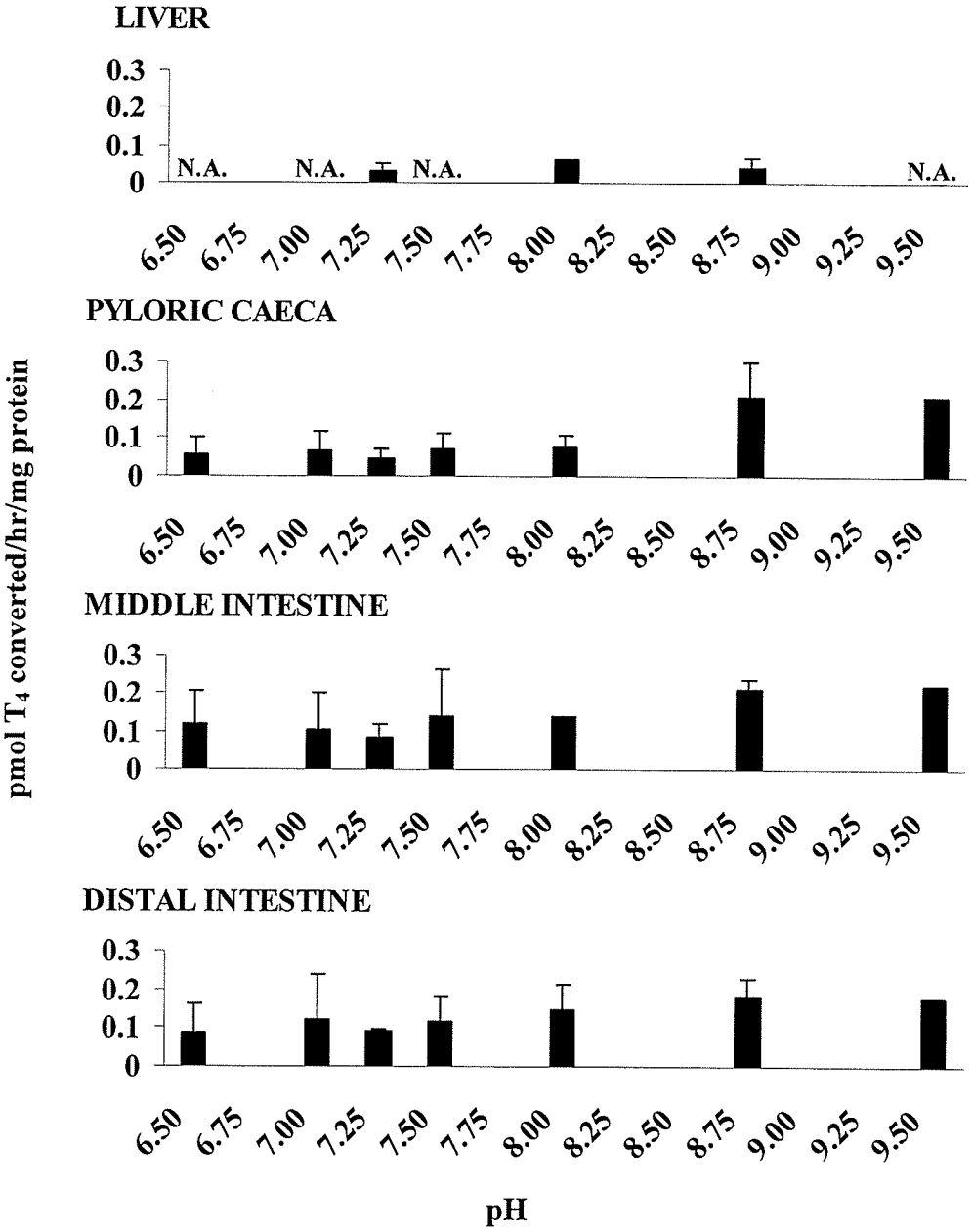


Figure 3-11. Rate of total T_4 metabolism (presumed $*I^-$ and/or *IP) without DTT over a range of pH values (6.5, 7.0, 7.2, 7.5, 8.0, 8.8, or 9.5) as determined by LH-20 column chromatography after $*T_4$ incubation with liver (n=1-2), pyloric caeca (n=1-6), middle intestine (n=1-5), or distal intestine (n=1-5) microsomes. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein (±s.e.). N.A. denotes not assayed.

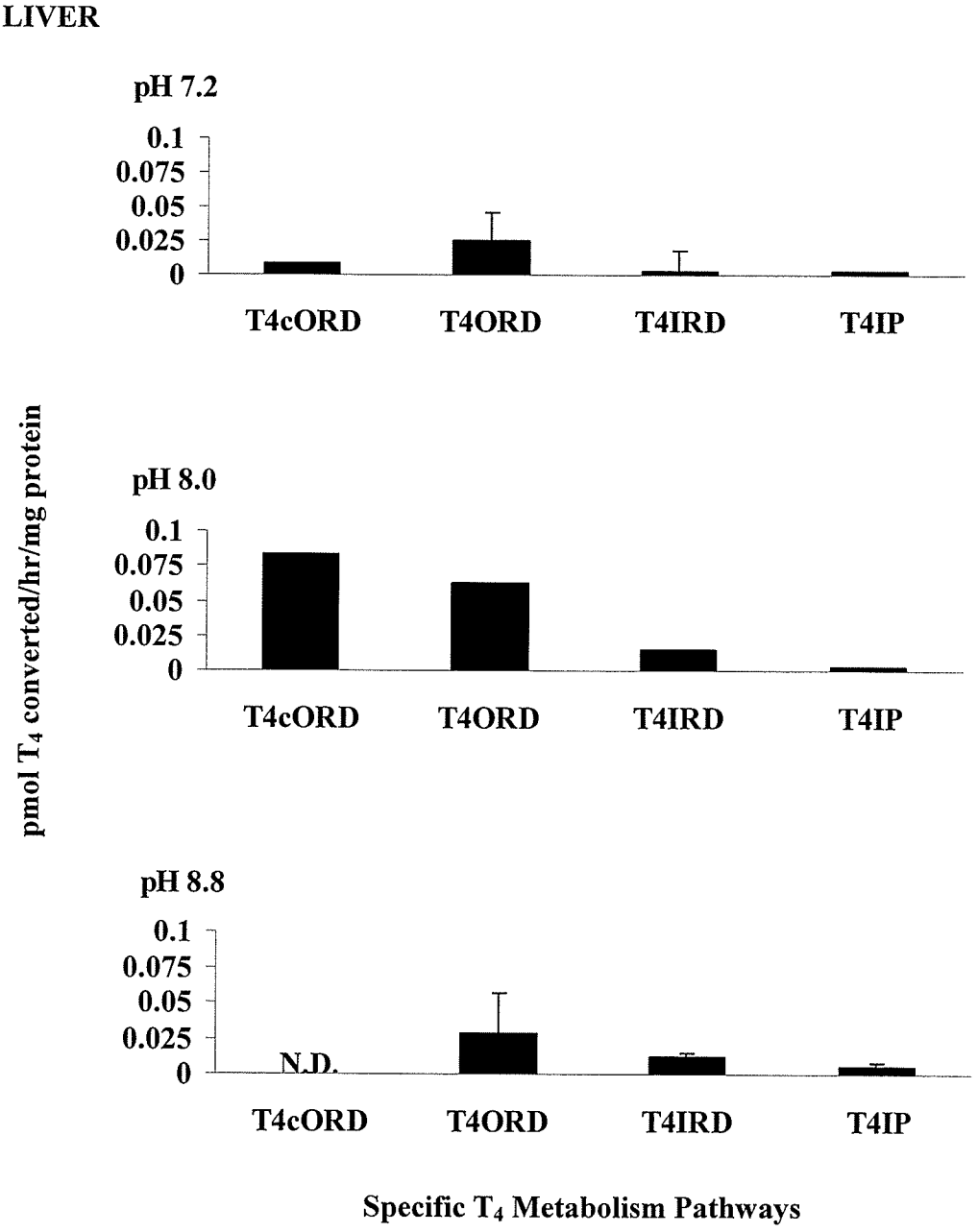


Figure 3-12. T_4cORD, T_4ORD, and T_4IRD activities without DTT at pH of 7.2 (n=3), 8.0 (n=1), or 8.8 (n=2) as determined by HPLC separation of $*I^-$, $*T_3$, and $*rT_3$ as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_4$ incubation with liver microsomes. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein (±s.e.). N.A. denotes not assayed. N.D. denotes non-detectable.

PYLORIC CAECA

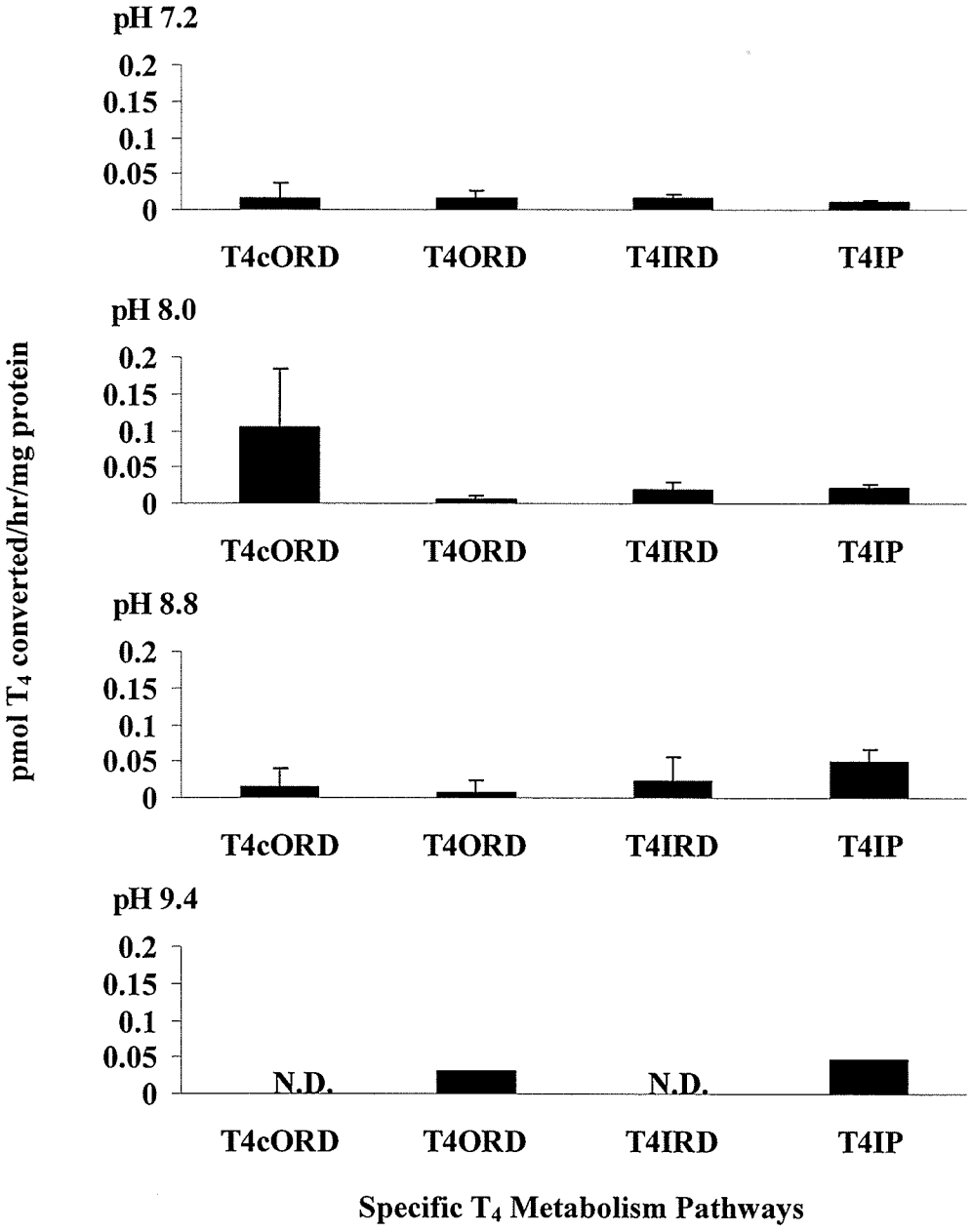


Figure 3-13. T_4cORD, T_4ORD, and T_4IRD activities without DTT at pH of 7.2 (n=3), 8.0 (n=4), 8.8 (n=4), or 9.4 (n=1) as determined by HPLC separation of $*I^-$, $*T_3$, and $*rT_3$ as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_4$ incubation with pyloric caeca microsomes. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein (±s.e.). N.D. denotes non-detectable.

MIDDLE INTESTINE

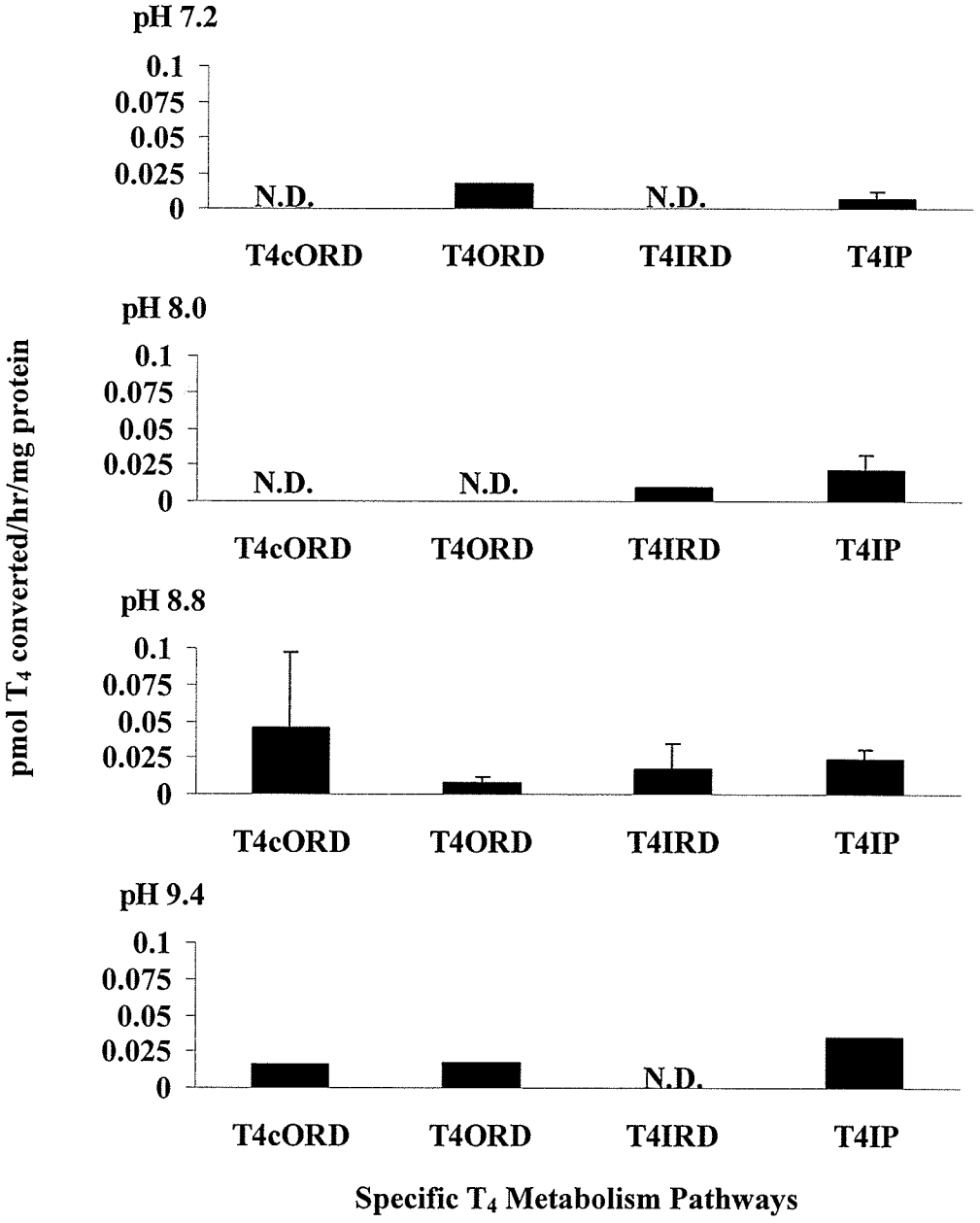


Figure 3-14. T_4cORD, T_4ORD, and T_4IRD activities without DTT at pH of 7.2 (n=5), 8.0 (n=4), 8.8 (n=2), or 9.4 (n=1) as determined by HPLC separation of *I⁻, *T_3, and *rT_3 as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after *T_4 incubation with middle intestine microsomes. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein (±s.e.). N.D. denotes non-detectable.

DISTAL INTESTINE

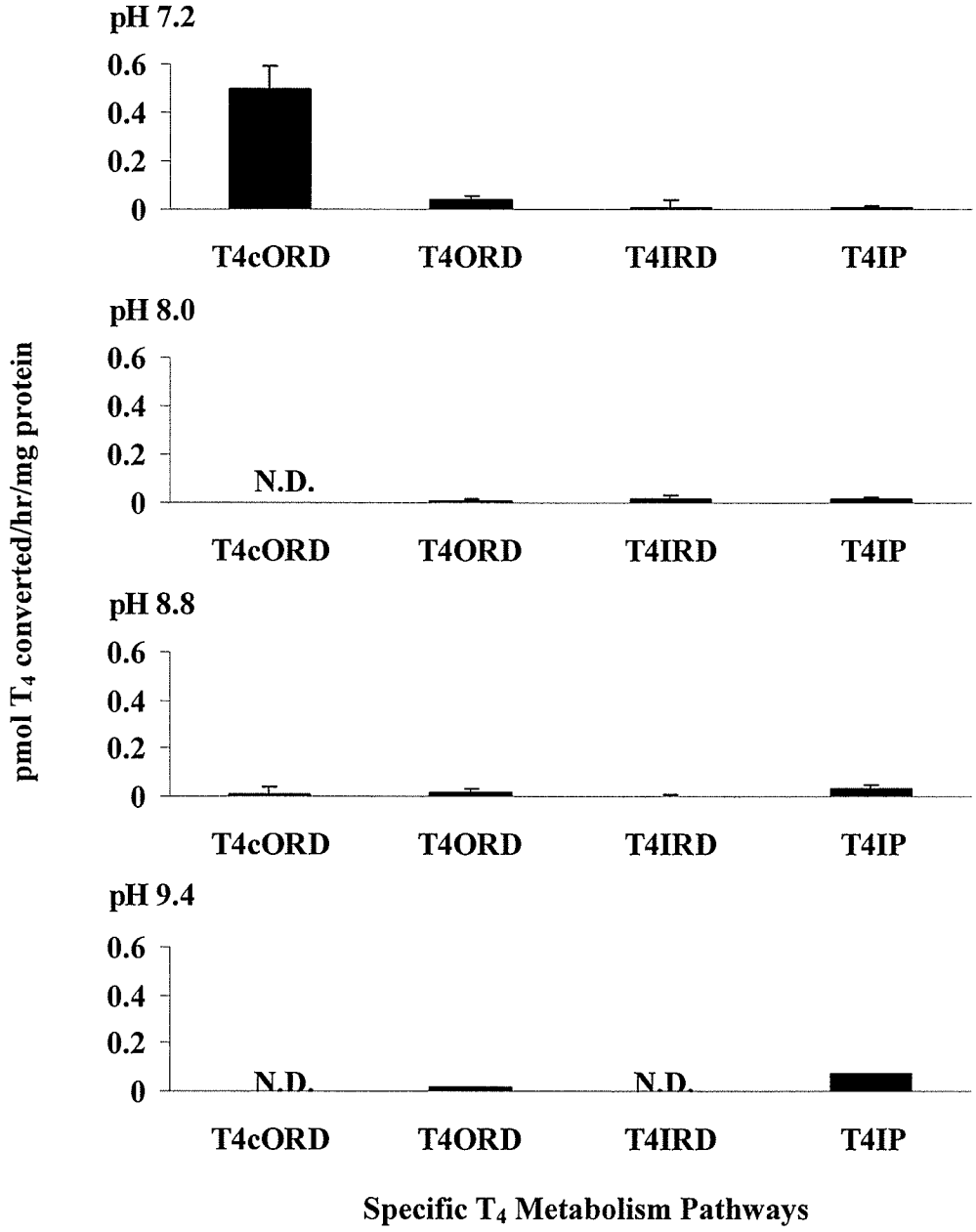


Figure 3-15. T_4cORD, T_4ORD, and T_4IRD activities without DTT at pH of 7.2 (n=5), 8.0 (n=2), 8.8 (n=2), or 9.4 (n=1) as determined by HPLC separation of $*I^-$, $*T_3$, and $*rT_3$ as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_4$ incubation with distal intestine microsomes. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein (±s.e.). N.D. denotes non-detectable.

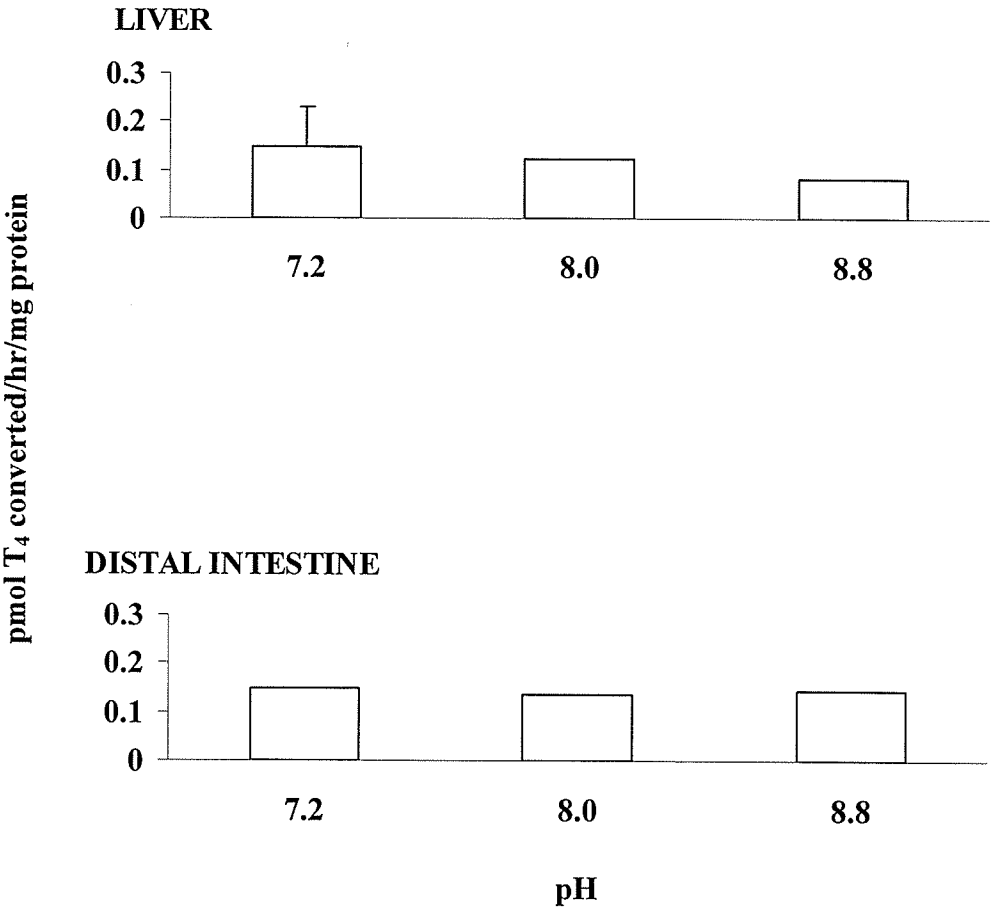


Figure 3-16. Rate of total T_4 metabolism (presumed $*I^-$ and/or *IP) at DTT concentration of 10 mM over a range of pH 7.2, 8.0, or 8.8 as determined by LH-20 column chromatography after $*T_4$ incubation with liver (n=1-2) or distal intestine (n=1) microsomes. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein (± s.e.).

LIVER

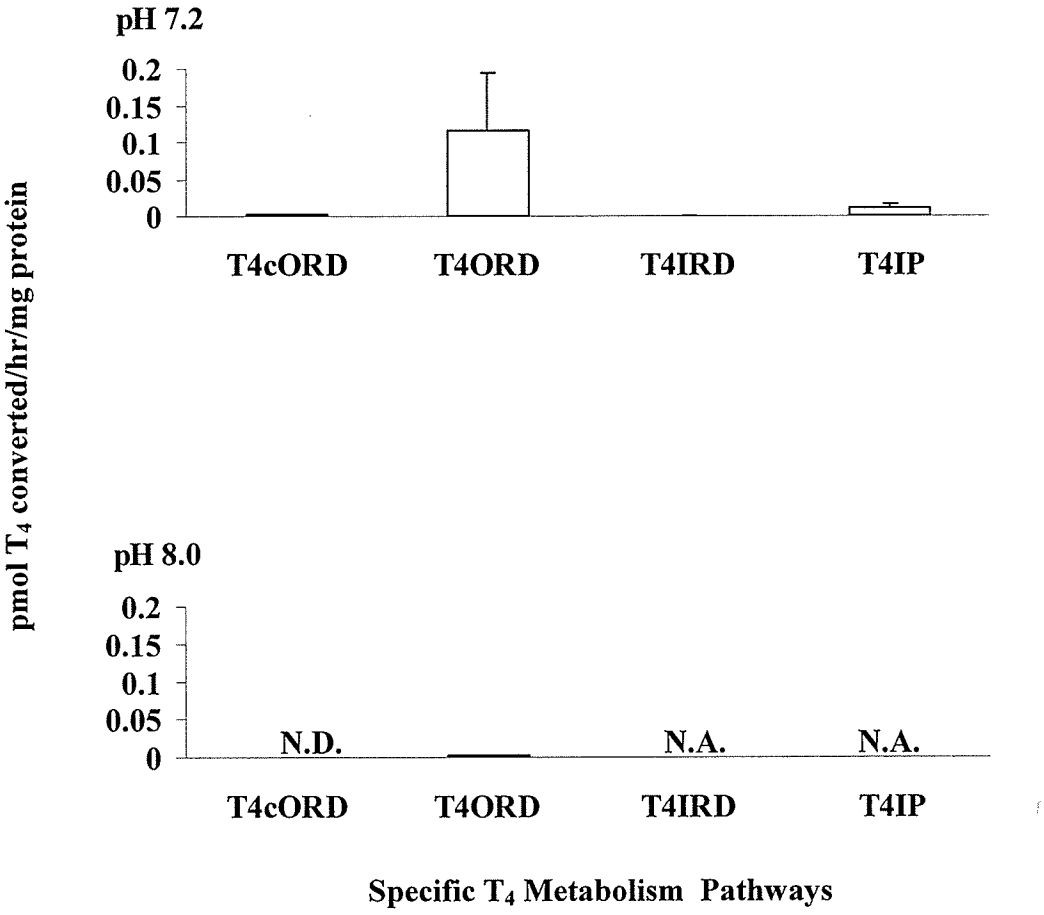


Figure 3-17. T_4cORD, T_4ORD, and T_4IRD activities at DTT concentration of 10 mM at pH of 7.2 (n=4) or 8.0 (n=3) as determined by HPLC separation of *I⁻, *T_3, and *rT_3 as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after *T_4 incubation with liver. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein (±s.e.). N.A. denotes not assayed. N.D. denotes non-detectable.

DISTAL INTESTINE

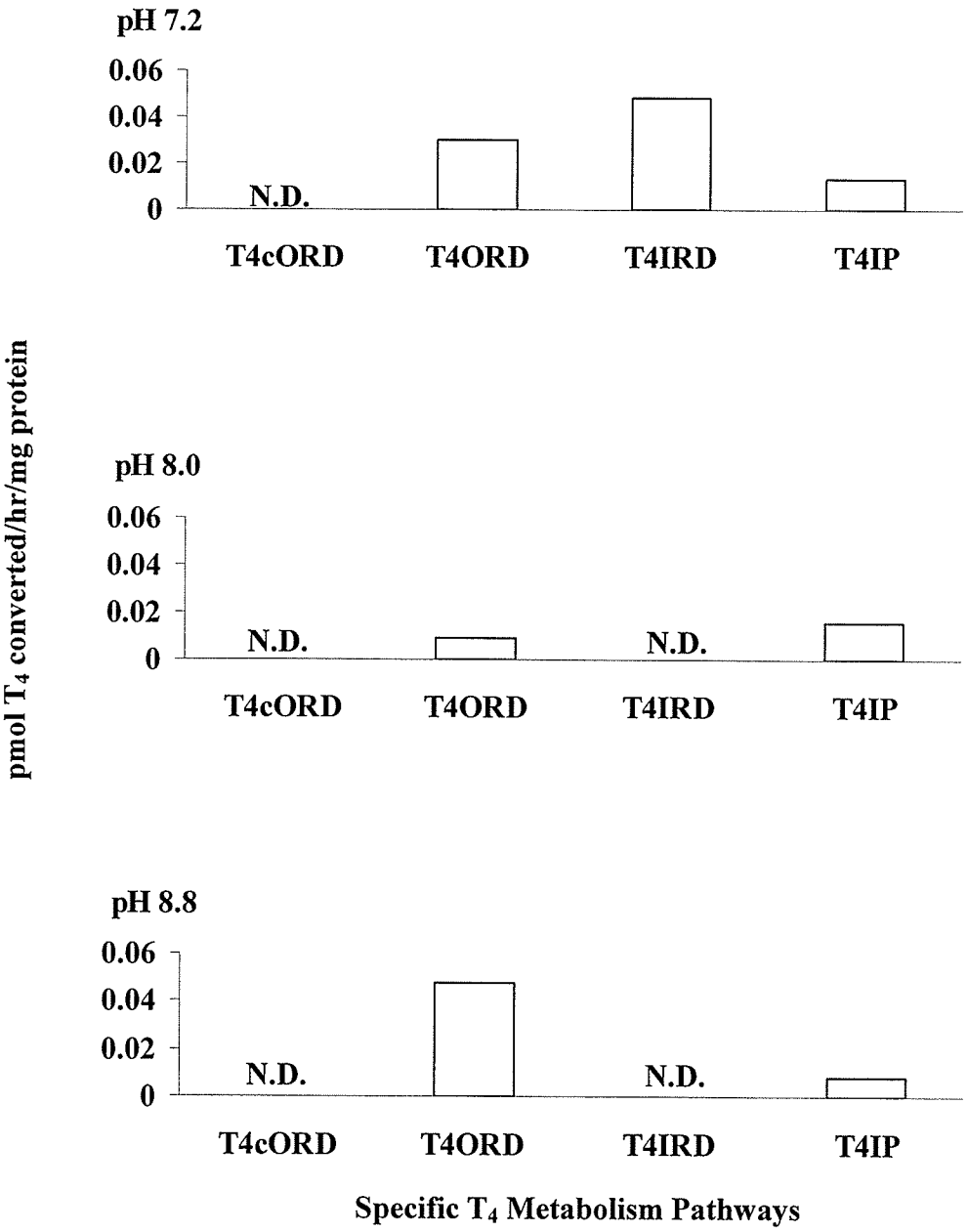


Figure 3-18. T_4cORD, T_4ORD, and T_4IRD activities at DTT concentration of 10 mM at pH of 7.2 (n=1), 8.0 (n=1), or 8.8 (n=1) as determined by HPLC separation of $*I^-$, $*T_3$, and $*rT_3$ as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_4$ incubation with distal intestine microsomes. Activities were calculated as pmoles of T_4 converted to product per hour per milligram of microsomal protein. N.D. denotes non-detectable.

LIVER

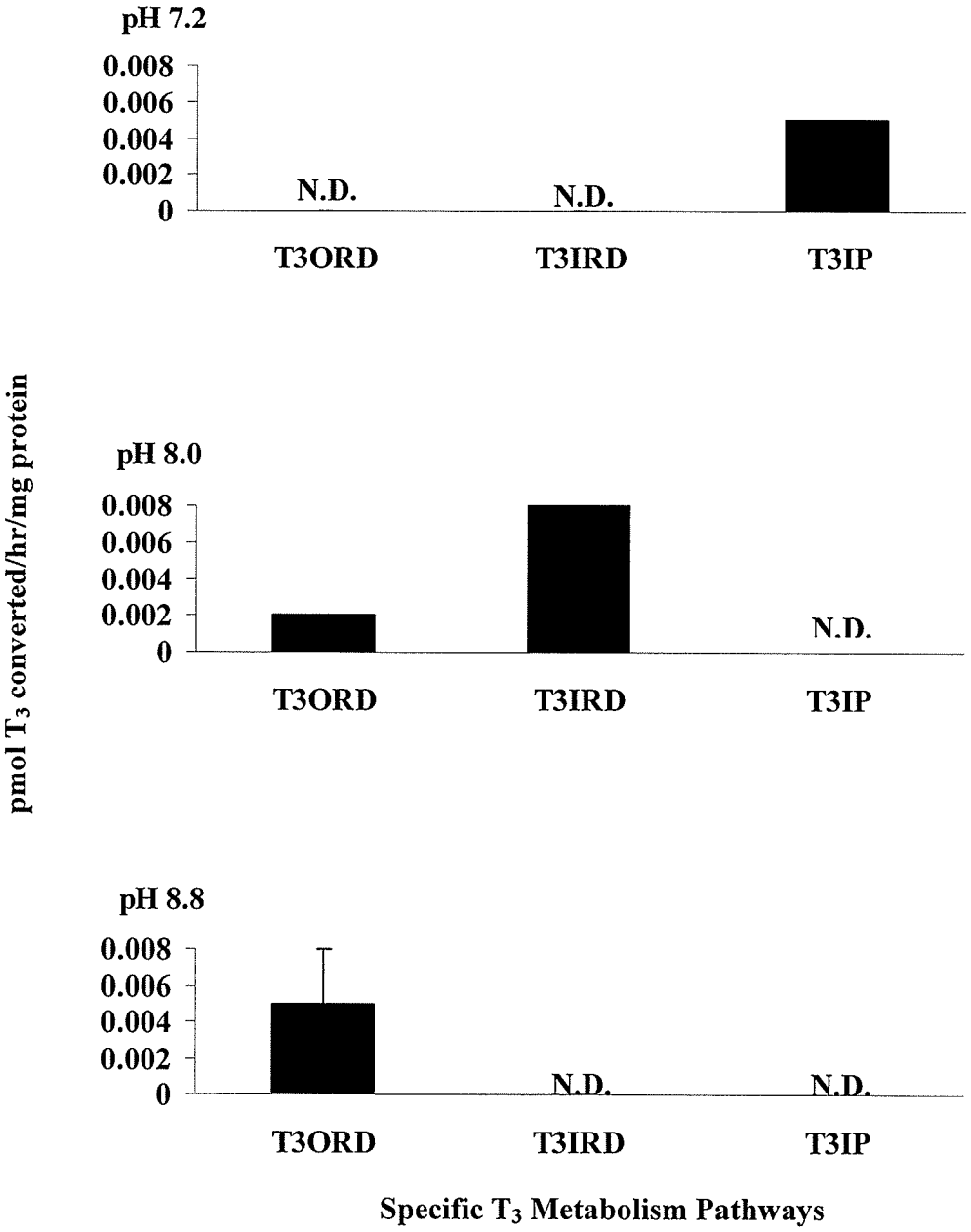


Figure 3-19. T_3ORD and T_3IRD activities without DTT at pH of 7.2 (n=1-3), 8.0 (n=1), or 8.8 (n=3) as determined by HPLC separation of $*I^-$ and $*T_2$, as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_3$ incubation with liver microsomes. Activities were calculated as pmoles of T_3 converted to product per hour per milligram of microsomal protein (± s.e.). N.D. denotes non-detectable.

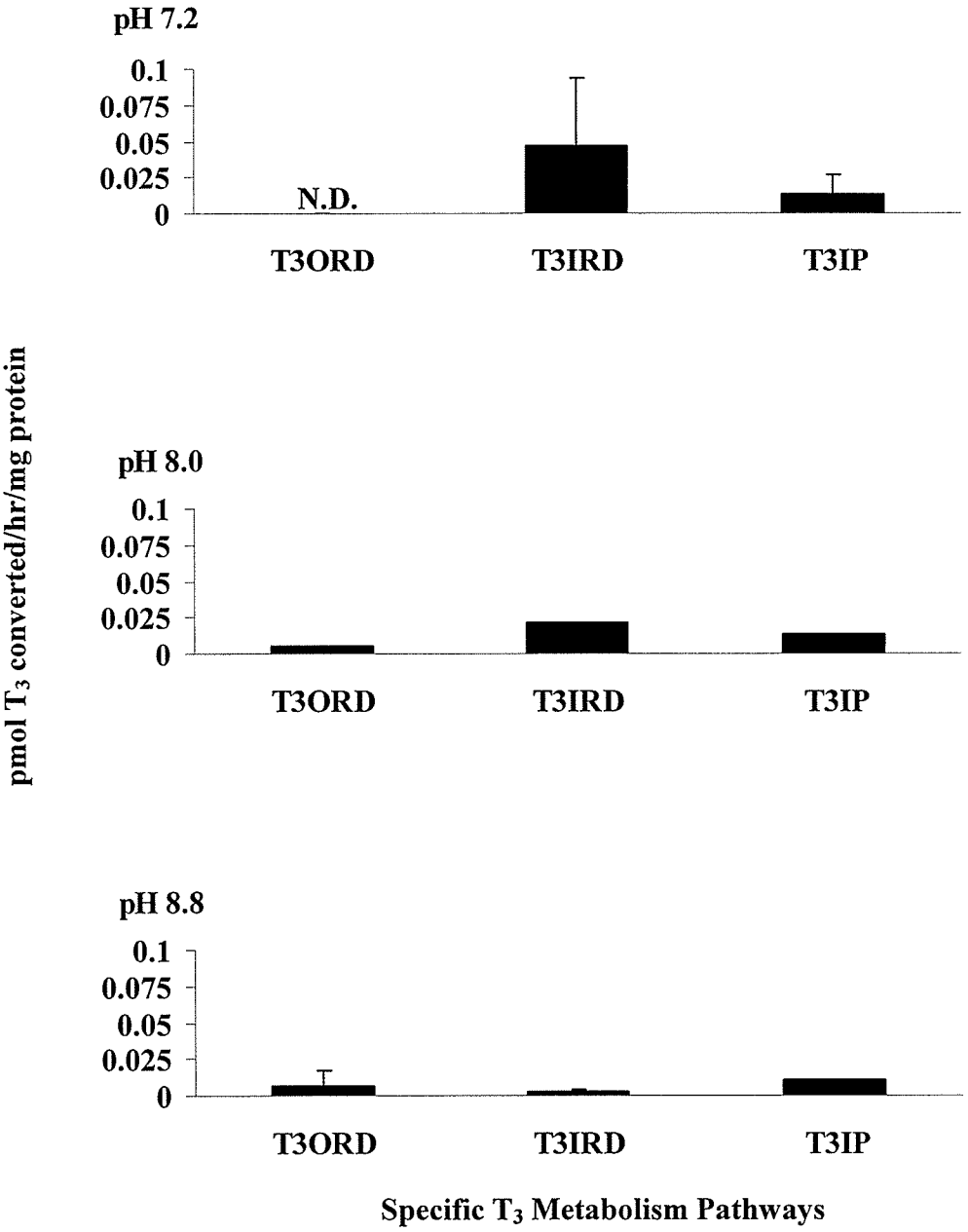


Figure 3-20. T_3ORD and T_3IRD activities without DTT at pH of 7.2 (n=4), 8.0 (n=1), or 8.8 (n=2) as determined by HPLC separation of $*I^-$ and $*T_2$, as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_3$ incubation with pyloric caeca microsomes. Activities were calculated as pmoles of T_3 converted to product per hour per milligram of microsomal protein (±s.e.). N.D. denotes non-detectable.

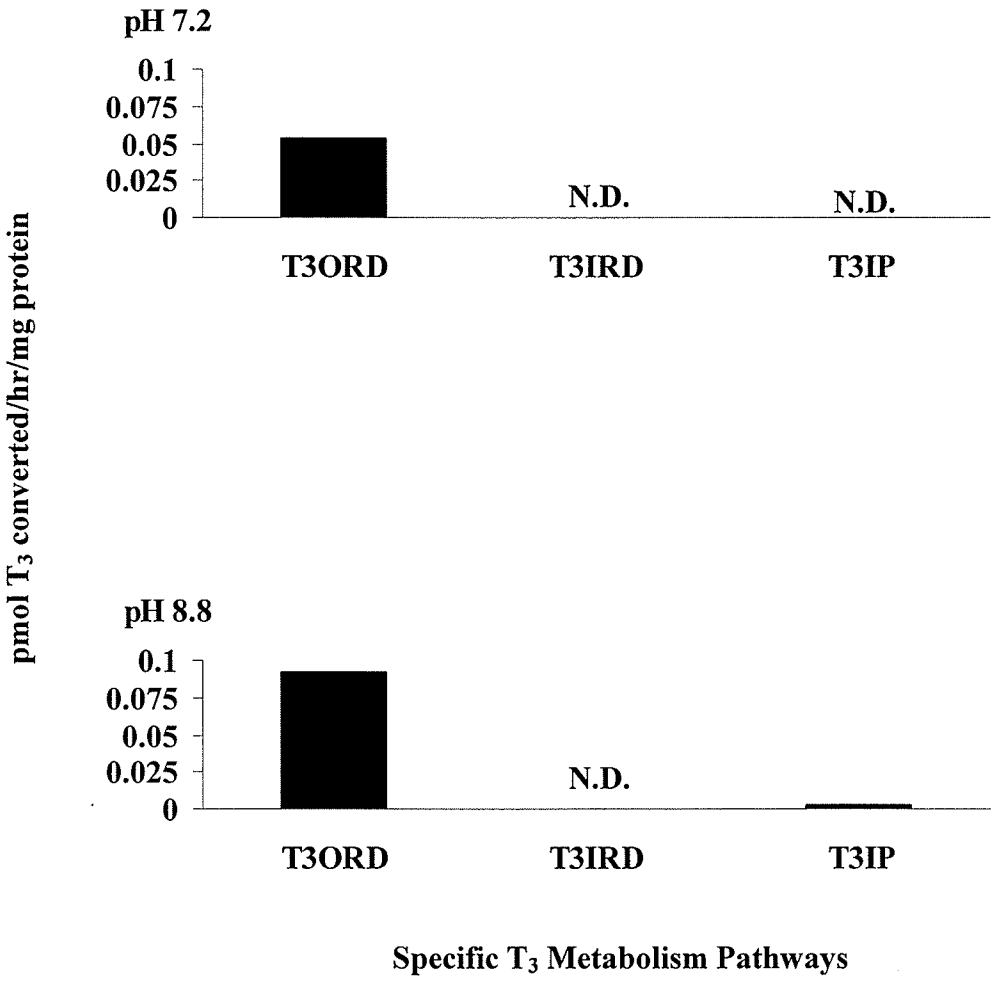


Figure 3-21. T_3ORD and T_3IRD activities without DTT at pH of 7.2 (n=1), 8.0 (n=1), or 8.8 (n=1) as determined by HPLC separation of $*I^-$ and $*T_2$, as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_3$ incubation with middle intestine microsomes. Activities were calculated as pmoles of T_3 converted to product per hour per milligram of microsomal protein (±s.e.). N.A. denotes not assayed. N.D. denotes non-detectable.

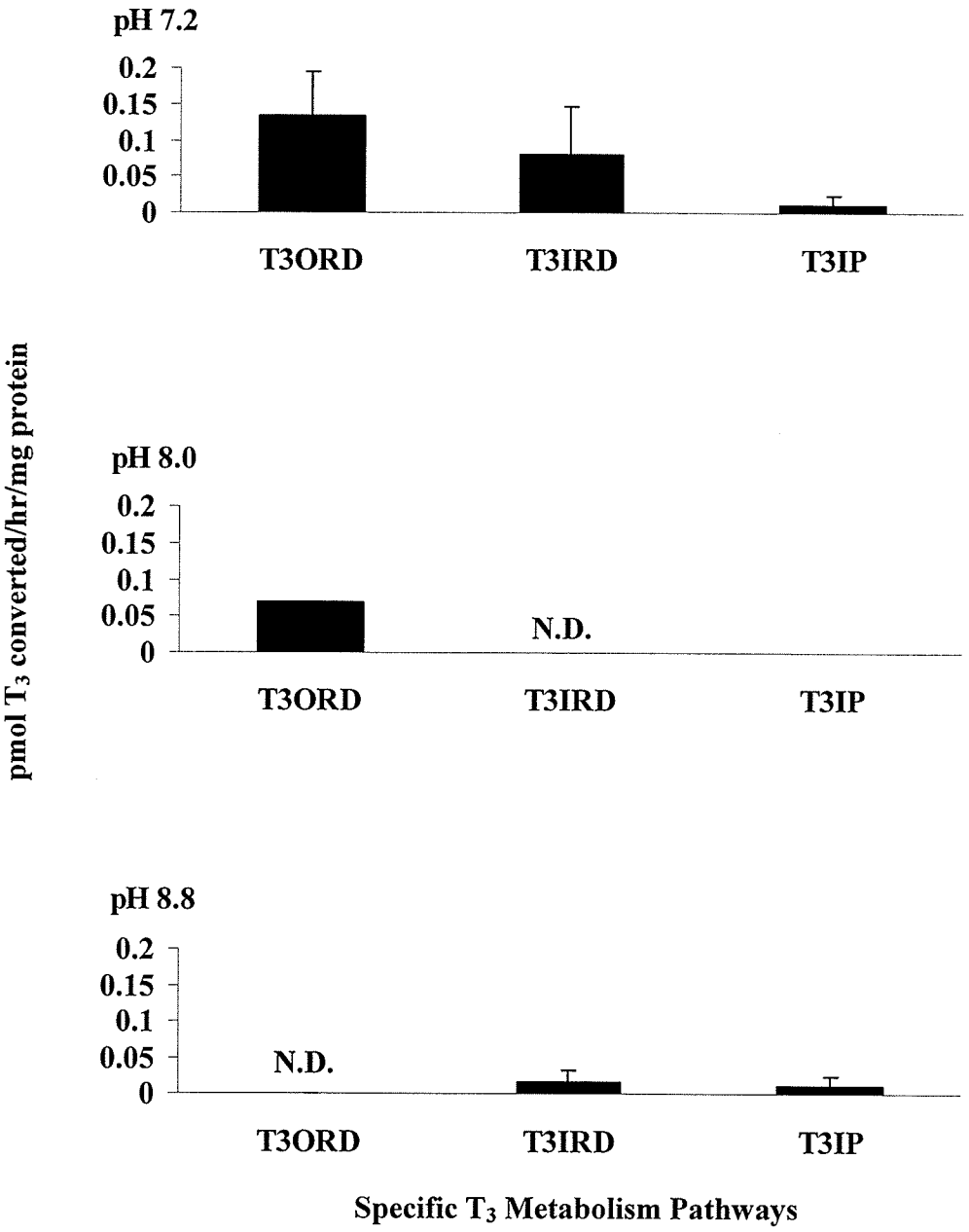


Figure 3-22. T_3ORD and T_3IRD activities without DTT at pH of 7.2 (n=3), 8.0 (n=1), or 8.8 (n=2) as determined by HPLC separation of *I⁻ and *T_2, as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after *T_3 incubation with distal intestine microsomes. Activities were calculated as pmoles of T_3 converted to product per hour per milligram of microsomal protein (±s.e.). N.A. denotes not assayed. N.D. denotes non-detectable.

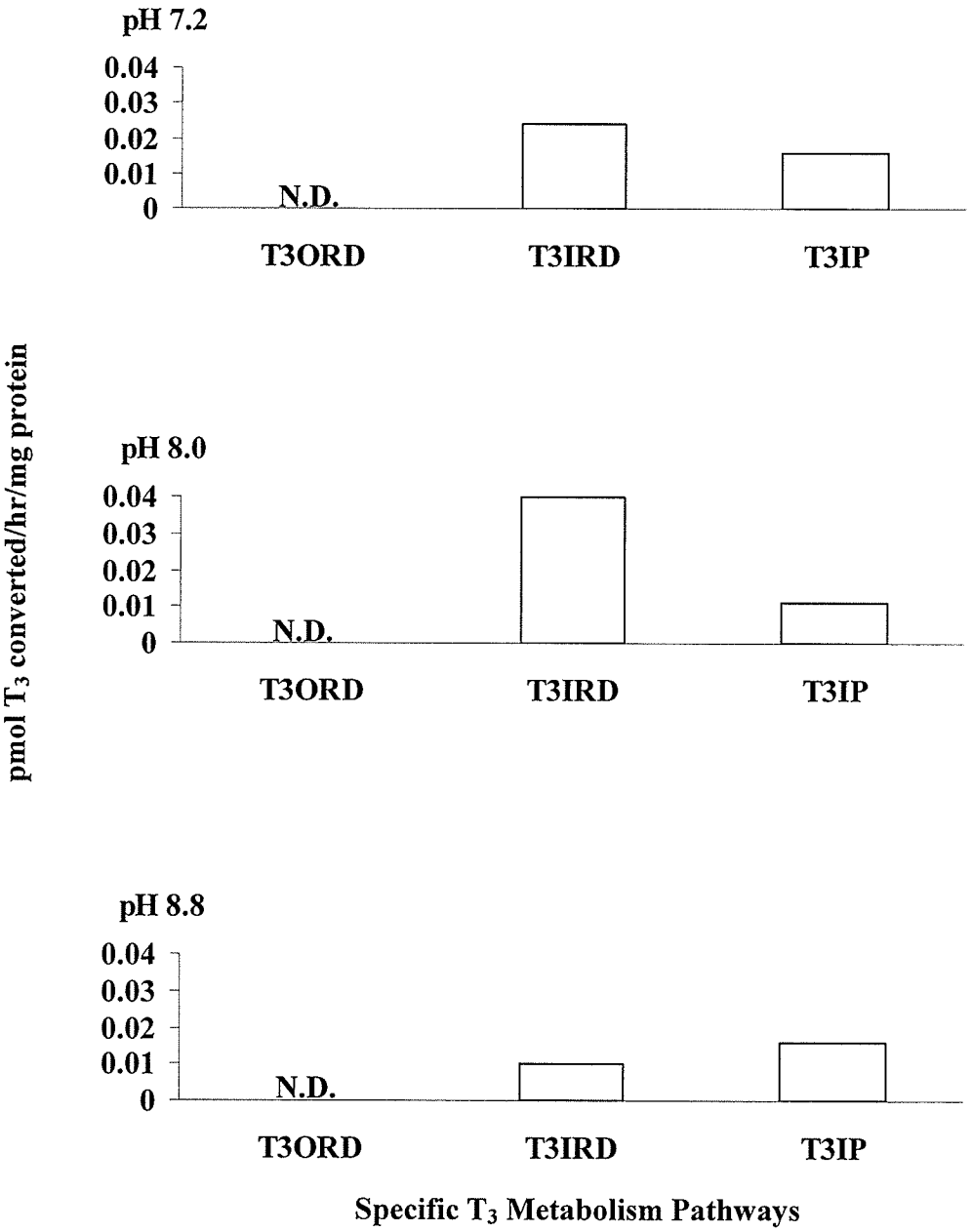


Figure 3-23. T_3ORD and T_3IRD activities at DTT concentration of 10 mM at pH of 7.2 (n=1), 8.0 (n=1), or 8.8 (n=1) as determined by HPLC separation of $*I^-$ and $*T_2$, as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_3$ incubation with liver microsomes. Activities were calculated as pmoles of T_3 converted to product per hour per milligram of microsomal protein. N.D. denotes non-detectable.

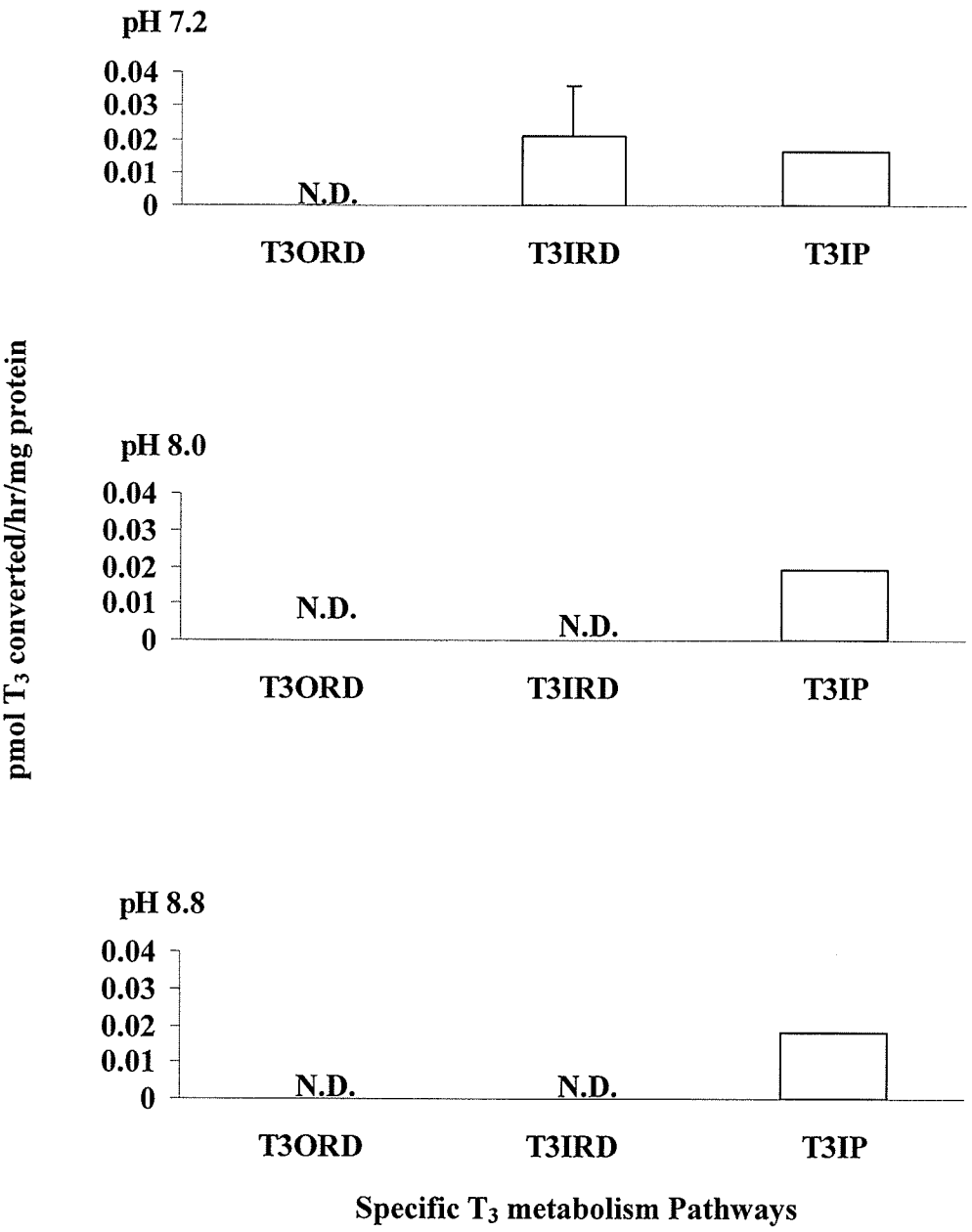


Figure 3-24. T_3ORD and T_3IRD activities at DTT concentration of 10 mM at pH of 7.2 (n=3), 8.0 (n=1), or 8.8 (n=1) as determined by HPLC separation of $*I^-$ and $*T_2$, as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_3$ incubation with pyloric caeca microsomes. Activities were calculated as pmoles of T_3 converted to product per hour per milligram of microsomal protein (±s.e.). N.D. denotes non-detectable.

DISTAL INTESTINE

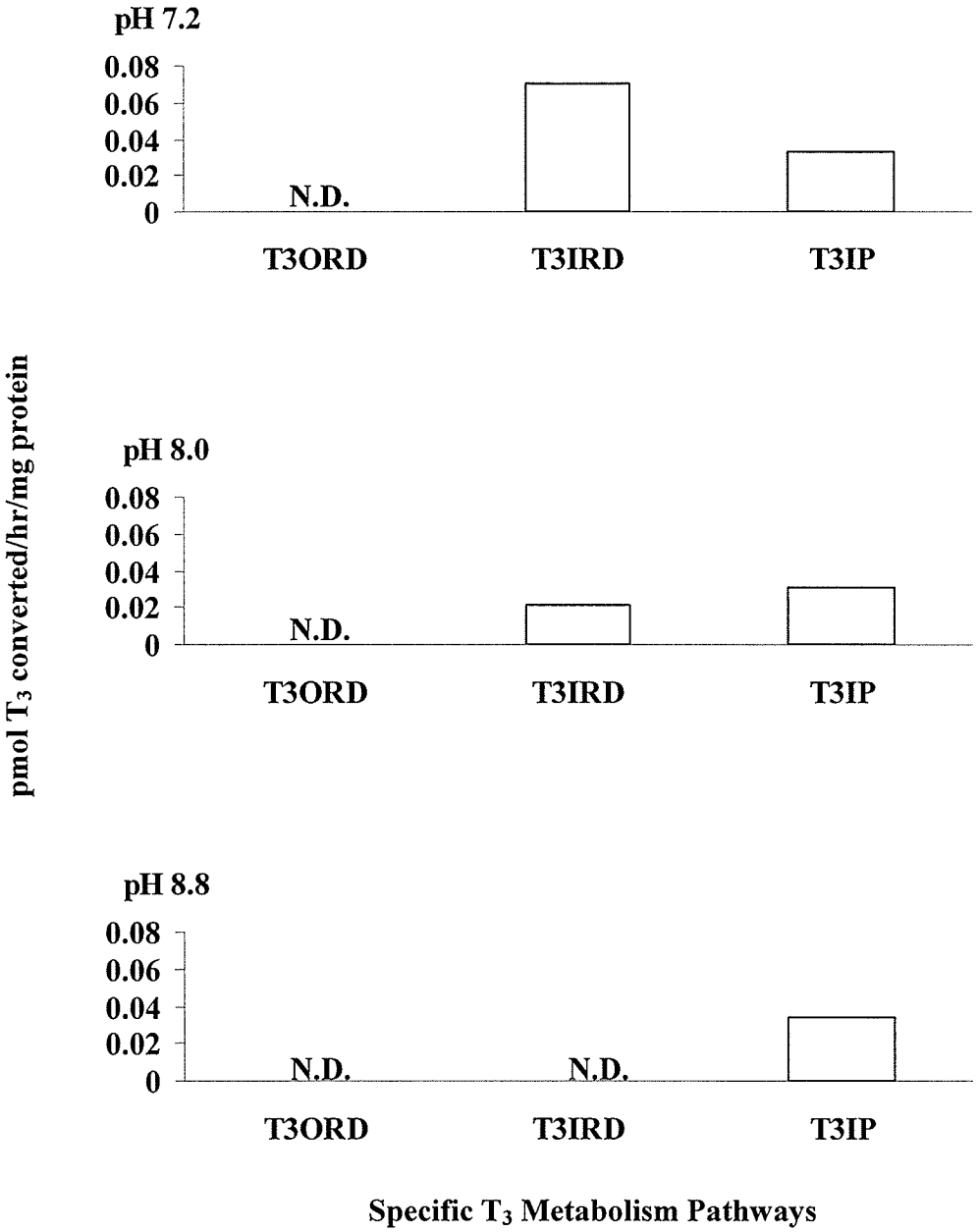


Figure 3-25. T_3ORD and T_3IRD activities at DTT concentration of 10 mM at pH of 7.2 (n=3), 8.0 (n=1), or 8.8 (n=1) as determined by HPLC separation of $*I^-$ and $*T_2$, as well as IP production as determined by LH-20 column chromatography followed by TCA precipitation of *IP after $*T_3$ incubation with distal intestine microsomes. Activities were calculated as pmoles of T_3 converted to product per hour per milligram of microsomal protein. N.D. denotes non-detectable.

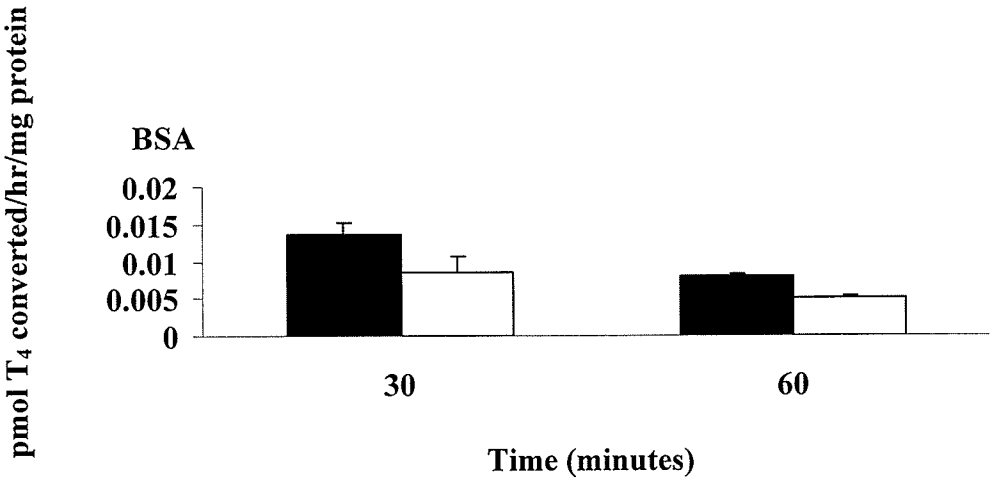


Figure 3-26. Rate of T_4IP production at DTT concentrations of 0 (■) or 20 (□) mM as determined by LH-20 column chromatography followed by TCA precipitation of *IP after *T_4 incubation with 0.2 mg/mL bovine serum albumin (BSA; n=4). Activities were calculated as pmoles of T_4 converted to IP per hour per milligram of BSA protein (±s.e.).

Chapter 4

Effects of Dietary Thyroid Hormone Challenges (T_4 and T_3) on Intestinal Metabolism of Thyroid Hormones in Rainbow Trout

Introduction

The basic pathways for thyroid hormone (TH) metabolism include deiodination, conjugation, deamination, ether-link cleavage, and covalent bonding to proteins (DiStefano, 1988; Hennemann, 1996). Of these, deiodination has been investigated the most due to its role in peripheral regulation of T_3 available to bind to receptors, or thyroidal status, in teleost fish (MacLatchy and Eales, 1993; Eales *et al.*, 1993; Eales, 1995).

In vitro liver, kidney, gill, and brain deiodination of TH has been described for rainbow trout (MacLatchy and Eales, 1992; Fines *et al.*, 1999). The liver, which is a main regulator of thyroidal status in rainbow trout, has also been investigated under various physiological states such as when circulating TH have been increased within physiological levels by feeding TH-supplemented food. With the administration of a TH challenge through the diet, the deiodination pathways were altered in the liver, favoring degradation of the hormones into inactive forms (Eales and Finnson, 1991; Sweeting and Eales, 1992; MacLatchy and Eales, 1993). Thus, liver studies have demonstrated autoregulation of T_3 homeostasis in a peripheral tissue.

In the previous chapter, the intestine was shown to metabolize TH. This in itself does not indicate a regulatory role of the intestine, especially if the intestine serves as an excretory pathway only. This study further investigated the phenomenon of peripheral

regulation by considering another tissue, the intestine, that would initially be exposed to the increased levels of TH before the liver. The intestine would be exposed to the heaviest onslaught of TH through direct contact with supplemented food. If intestinal TH metabolism pathways were regulated in response to various levels of TH, the intestine might also control the amounts of TH entering the circulation via the intestine. Thus, the intestine may regulate thyroidal status when faced with a persistent dietary load of TH that could increase plasma TH levels within physiological ranges.

To investigate the potential role of the intestine in regulating thyroidal status, TH metabolism pathways were monitored during dietary TH challenges. This was done by feeding TH (T_4 treatment and T_3 treatment) in the diet over a period of 7 days, and then measuring changes in plasma TH levels. A dietary T_3 challenge is known to increase plasma T_3 levels within physiological ranges (Higgs *et al.*, 1979; Higgs *et al.*, 1982; Cyr and Eales, 1990; Eales *et al.*, 1990; Eales and Finnson, 1991; Sweeting and Eales, 1992b; Fines *et al.*, 1999). In the present study, TH metabolism pathways in the intestinal regions (pyloric caeca, PC; middle intestine, MI; distal intestine, DI) were compared among controls, T_4-treated, and T_3-treated fish to see if pathways were modulated under dietary TH challenges. Thus, this chapter reports on investigations of the regulation of intestinal metabolism during a physiological challenge, and its possible role in regulation of thyroidal status in the rainbow trout.

Materials and Methods

Fish Maintenance

Rainbow trout were obtained from Rockwood Hatchery (Gunton, Manitoba) and held at the University of Manitoba in running, aerated, dechlorinated, City of Winnipeg water at 12°C and under a 12L:12D photocycle. Sexually immature trout (160-351 g; mean = 260.5 ± 5.7) were placed in 9 tanks (60-cm x 60-cm x 60-cm) with 15 trout per tank and acclimated for 2 weeks while fed commercial trout food diet (3.2 mm, Martin Mills) once daily at 1% of body mass.

Dietary T_4 and T_3 challenges

For the next 7 days, trout were fed either food sprayed with methanol alone (control; 3 tanks), food sprayed with T_4 dissolved in methanol to provide 12 ppm T_4 (T_4 treatment; 3 tanks), or food sprayed with T_3 dissolved in methanol to provide 12 ppm T_3 (T_3 treatment; 3 tanks).

Sampling

At Day 0 (1 day before treatment), Day 2, Day 4, and Day 7, 3 trout per tank were removed for sampling (n=9 per treatment per sampling day). Trout were anesthetized with tricaine methanesulfonate (MS222, 0.067g/L, Syndel Labs), killed by a blow to the head, bled from caudal blood vessels using heparinized syringes, and the liver, PC, MI, and DI were removed. Each tissue was frozen in liquid nitrogen and stored at -76°C. Blood samples were centrifuged at 17,000 g for 5 min, following which the plasma was removed and stored in Eppendorf tubes at -76°C until analysis.

Plasma T_4 and T_3 levels

Plasma T_4 and T_3 levels were determined for control, T_4 treatment, and T_3 treatment for Days 0, 2, and 7 using solid-phase radioimmunoassays (RIA) modified from Brown and Eales (1977), Omeljaniuk *et al.* (1984), and Kohel *et al.* (2001). Separate T_4 and T_3 RIA were performed on G-25 Sephadex columns. The samples were diluted in 100 µL buffer (T_4 RIA barbital buffer: 12.15 g barbital per L H_2O, pH 8.6; T_3 RIA phosphate buffer: 26.81 g $Na_2HPO_4 \cdot 7\ H_2O$, 11.17 g Na_2EDTA per 1 L H_2O, pH 7.2)/8-anilo-1-napthelene sulfonic acid (ANS; 1 mg ANS/1 mL buffer). 100 µL $*T_4$ or $*T_3$ (approximately 20,000 cpm/100 µL buffer) was incubated with the plasma sample overnight. The free iodide (I^-) was eluted with 3 mL buffer. The TH was eluted with 1 mL T_4 antibody (Sigma; diluted in barbital buffer) or 1 mL T_3 antibody (Sigma; diluted in phosphate buffer) and counted in the gamma counter.

Microsomal fraction preparations

Microsomes were prepared from individual liver, PC, MI, and DI tissues following the procedure of Shields and Eales (1986), as described in Chapter 3.

TH metabolism assay

The TH metabolism assay was performed on samples collected from Days 0, 2, and 7. The assay was based on the microsomal TH deiodinase assay methods of Shields and Eales (1986) and Frith and Eales (1996). Details of the modified procedures were described in Chapter 3. Each TH metabolism assay was run in duplicate under the following conditions: 30 min preincubation in a shaking water bath (12°C, 140 rpm, darkness), 0 mM or 10 mM DTT cofactor, pH 7.2, and 45 min incubation time with either

0.16 nM T_4 or 0.19 nM T_3 substrate and the corresponding radioactive TH (*TH; approximately 100,000 cpm/sample).

TH metabolism analysis

The incubate was separated into two aliquots. One aliquot was used to determine specific TH metabolism products (T_4cORD, T_4ORD, and T_4IRD; T_3ORD and T_3IRD) by using HPLC separation as described in Chapter 3 (Figure 3-3 and 3-4). The other aliquot was used to determine IP production (T_4IP or T_3IP) by using LH-20 column separation and protein precipitation as described in Chapter 3 (Figure 3-3). Activities were expressed as picomoles of T_4 or T_3 converted to product per hour per milligram of microsomal protein. Detailed description of TH metabolism products (Figure 1-2) and analyses (Figure 3-4) are found in Chapter 3.

Statistics

Using the SPSS statistics package, univariate analysis of variance (ANOVA) was used to compare plasma T_4 means for the effects of treatments (C, T_4 TRT, and T_3 TRT) and to compare plasma T_3 means for the effects of treatments. Each test was performed for individual days. These were followed by LSD post hoc multiple comparisons to determine significant differences among treatments. Results were determined statistically significant at $p \leq 0.05$.

Linear regression analyses were computed to compare TH metabolism activities for the effects of treatments (C, T_4 TRT, and T_3 TRT) and time (Day 0, 2, and7) for both 0 mM and 10 mM DTT, individually. Tukey's Multiple Comparison was used to compute confidence intervals and determine significant differences among treatments

over time (see Appendix IX for equations used). Results were determined statistically significant at $p \leq 0.05$.

Results

Effects of dietary T_4 and T_3 challenges on plasma T_4 and T_3 levels

Neither dietary T_4 nor T_3 challenges significantly changed plasma T_4 levels by Day 7 (Figure 4-1; F=0.462, d.f.=2, p=0.635).

A dietary T_3 challenge significantly increased plasma T_3 levels from 0.4 ng/mL on Day 0 to 3.1 ng/mL on Day 2 (F=8.043, d.f.=2, p=0.004), with a further increase to 5.1 ng/mL on Day 7 (F=25.303, d.f.=2, p=0.000). T_3 treatment did not, however, influence plasma T_4 levels (F=25.303. d.f.=2, p=0.540).

Effects of dietary T_4 and T_3 challenges on TH metabolism pathways

T_4cORD

At all times and DTT concentrations, T_4cORD was low or non-detectable in the liver. Without DTT, the highest levels of T_4cORD activity measured in controls occurred on Day 0 (0.17 pmol T_4 converted/hr/mg protein; Figure 4-2). With DTT, T_4cORD activity reached 0.24 pmol T_4 converted/hr/mg protein. No significant changes resulted from dietary T_4 or T_3 challenges.

In the intestine, the highest levels of T_4cORD activity were measured on Day 0. Of the intestinal regions studied, the DI exhibited the highest levels of T_4cORD activity throughout the time course (up to 2.3 pmol T_4 converted/hr/mg protein). However, only the MI exhibited significant changes in activity at Day 7 due to dietary TH challenges (F=1.734, d.f.=8 $p \leq 0.05$). At this time, T_3 treatment increased T_4cORD activity to 0.68

pmol T_4 converted/hr/mg protein in comparison to controls (0.09 pmol T_4 converted/hr/mg protein). DI exhibited a similar pattern, however, the changes were not significant.

T_4ORD

Liver T_4ORD activity required DTT (Figure 4-3). By Day 7, a dietary T_3 challenge significantly decreased T_4ORD activity (0.03 pmol T_4 converted/hr/mg protein) from control levels (0.18 pmol T_4 converted/hr/mg protein; $F=1.400$, d.f.=8, $p \leq 0.05$). A dietary T_4 challenge did not affect T_4ORD activity.

Intestinal levels of T_4ORD activity were much lower than in the liver. The T_4ORD activity with DTT was not prominent in the intestine, except in the MI at Day 0. At Day 0, MI T_4ORD activity was higher with DTT (0.02 pmol T_4 converted/hr/mg protein) than without DTT (non-detectable). Otherwise, T_4ORD activity levels were low with or without DTT. At Day 7, only activity in the DI was affected by dietary TH challenges. T_4ORD activity was significantly higher after seven days of either T_4 or T_3 treatment (0.08 and 0.10 pmol T_4 converted/hr/mg protein, respectively; $F=1.779$, d.f.=8, $p \leq 0.05$). The increased T_4ORD activity did not require DTT.

T_4IRD

In the liver, highest levels were measured at Day 0 in controls (0.09 pmol T_4 converted/hr/mg protein; Figure 4-4). T_4IRD activity increased significantly with T_3 treatment after 7 days to 0.04 pmol T_4 converted/hr/mg protein from 0.002 pmol T_4 converted/hr/mg protein in controls ($F=3.751$, d.f.=8, $p \leq 0.05$).

A similar pattern of increased T_4IRD activity with T_3 treatment at Day 7 was seen in the PC (0.03 pmol T_4 converted/hr/mg protein, T_3 treatment; 0.001 pmol T_4

converted/hr/mg protein, control; F=2.564, d.f.=8, $p \leq 0.05$) and the MI (0.04 pmol T_4 converted/hr/mg protein, T_3 treatment; 0.01 pmol T_4 converted/hr/mg protein, control; F=1.150, d.f.=8, $p \leq 0.05$). In these intestinal regions, T_4IRD activity required DTT. In the DI, however, T_4IRD activity was elevated for all conditions at Day 7 (range: 0.06-0.10 pmol T_4 converted/hr/mg protein). The increased T_4IRD activity did not require DTT.

T_4IP

For the liver, levels of T_4IP production were low without and with DTT (range over 7 days: 0.002-0.01 pmol T_4 converted/hr/mg protein), and they did not change significantly at Day 7 with dietary TH challenges (Figure 4-5).

For intestinal regions, T_4IP production was highest at Day 0, with the highest activity in the DI (0.06 pmol T_4 converted/hr/mg protein). Yet, T_4IP was low by Day 7. T_4IP production was greater without DTT for all regions of the intestine. Only the MI significantly increased T_4IP production to 0.02 pmol T_4 converted/hr/mg protein after a dietary T_3 challenge by Day 7 from control levels of 0.006 pmol T_4 converted/hr/mg protein (F=2.354, d.f.=8, $p \leq 0.05$). A dietary T_4 challenge had no effect.

T_3ORD

In the liver, T_3ORD activity was low or non-detectable at all days sampled and at all levels of DTT (range over 7 days: 0.002-0.02 pmol T_3 converted/hr/mg protein; Figure 4-6).

In intestinal tissues, T_3ORD activity was also low, and highest levels were at Day 0 in controls for the PC and the DI (up to 0.6 pmol T_3 converted/hr/mg protein). In the MI, activity significantly increased at Day 7 with T_3 treatment (0.19 pmol T_3

converted/hr/mg protein; F=1.238, d.f.=8, $p \leq 0.05$). T_3ORD activity did not require DTT in the intestine.

T_3IRD

In the liver, T_3IRD activity was highest with DTT at Day 0 in controls (0.06 pmol T_3 converted/hr/mg protein; Figure 4-7). T_3IRD activity was low except for T_3 treatment at Day 2 and 7, where activity increased to 0.02 pmol T_3 converted/hr/mg protein. T_3IRD activity required DTT.

In the intestine, T_3IRD activity was also highest at Day 0 in controls (up to 0.09 pmol T_3 converted/hr/mg protein for DI). Otherwise, T_3IRD activity was low or non-detectable except in the DI. By Day 7, a dietary T_3 challenge significantly increased T_3IRD activity in the DI to 0.04 pmol T_3 converted/hr/mg protein from 0.004 pmol T_3 converted/hr/mg protein in controls (F=2.143, d.f.=8, $p \leq 0.05$). The T_3IRD activity required DTT.

T_3IP

In the liver, T_3IP production was very low to non-detectable (Figure 4-8).

In the intestine, T_3IP production was highest at Day 0 in controls, especially for the PC and the DI (0.1 pmol T_3 converted/hr/mg protein). After Day 0, T_3IP production was low or non-detectable, except in the DI at Day 2. T_3IP production declined to very low or non-detectable levels by Day 7. For all regions of the intestine, DTT was not required for T_3IP production.

Discussion

This chapter set out to describe alterations in intestinal TH metabolism under physiological TH challenges. Using the microsomal TH metabolism assay developed in Chapter 3, active TH metabolism pathways were measured during dietary T_4 and T_3 challenges to determine if the intestine could regulate TH metabolism which would possibly control the TH entering into circulation.

Effects of dietary T_4 and T_3 challenges on plasma T_4 and T_3 levels

A dietary T_4 challenge did not elevate plasma T_4, yet a dietary T_3 challenge significantly elevated plasma T_3. The purpose of feeding TH over a period of 7 days was to create physiological T_4 or T_3 challenges and to determine their effects on compensatory TH metabolism in intestinal tissues. A dietary TH challenge can alter the physiological condition of the trout (Higgs *et al.*, 1979), which influences regulation of TH (Eales *et al.*, 1993). For this study, the method of assessing the physiological challenge on the trout thyroidal status was to measure plasma TH levels. It was anticipated that a dietary TH challenge would elevate plasma TH levels. However, only a dietary T_3 challenge affected plasma T_3 levels.

It is possible that a dietary T_4 challenge was not a physiological challenge to the trout thyroidal status, especially if the stable plasma T_4 levels represented a lack of absorption of T_4 from the intestine. Previous studies indicated that T_4, in comparison to T_3, is poorly absorbed from the intestine, however these conclusions are based on indirect measurements such as plasma TH levels (Whitaker and Eales, 1993) or by measuring physiological parameters such as growth, food consumption, and food conversion which indicated that dietary T_3 has a greater effect than dietary T_4 (Higgs *et al.*, 1979). The

assessment of TH as a physiological challenge is incomplete without direct measurements of absorption or clearance pathways for TH.

However, the significance of a dietary T_4 challenge as a physiological challenge to thyroidal status should not be dismissed based on plasma T_4 levels. A lack of a significant increase in plasma T_4 may be an indicator of the sensitivity of plasma T_4 control. In trout, T_4 is regulated independently from T_3 (Eales and Himick, 1991; Eales *et al.*, 1993; Eales and Brown, 1993; Eales, 1995). T_4 is the main TH released from the thyroid (Eales and Brown, 1993). Secretion and its resultant plasma T_4 levels are regulated by negative feedback at the hypothalamus (Peter 1971, 1972) and the pituitary (Baker 1965, 1969a, b; Sage 1968; Sage and Bromage, 1970). On the other hand, T_3 availability is peripherally regulated by tissue deiodination, and this is dependent on tissue T_3 demand, not on T_4 availability (MacLatchy and Eales, 1993; Eales *et al.*, 1993). Independent regulation of TH was further supported by this experiment. A dietary T_4 challenge did not affect plasma T_3 levels, and likewise, a dietary T_3 challenge did not affect plasma T_4 levels. Thus, an increase in plasma T_4 due to absorption from the intestine could result in a compensatory decrease in thyroidal T_4 output. Unless the rate of absorption from the intestine and uptake into systemic circulation surpasses the thyroid's capacity to reduce T_4 output (along with systemic clearance rates), plasma T_4 levels may not be a reliable indicator of the resulting physiological condition following a dietary, or any, T_4 challenge.

A dietary T_3 challenge resulted in increased plasma T_3 levels. Dietary T_3 challenges have been shown to alter physiological states of coho salmon (Higgs *et al*, 1979). Fish growth, food consumption, and food conversion were altered with a range of

dietary T_3 challenges (20-500 ppm). In addition, circulating T_3, regardless of source, altered TH metabolism pathways in the liver to maintain T_3 homeostasis (Eales *et al.*, 1993). Increased T_3 decreased hepatic T_4ORD (Eales *et al.*, 1990) and increased hepatic T_4IRD (Sweeting and Eales, 1992b) and T_3IRD (MacLatchy and Eales, 1993). Thus, a dietary T_3 challenge that increases plasma T_3 levels is a physiological challenge to thyroidal status.

Overall, a dietary T_3 challenge was a physiological challenge to thyroidal status as demonstrated by increased plasma T_3 levels. A dietary T_4 challenge may have resulted in a physiological challenge as well. However, plasma T_4 levels were not a good indicator. The consistent dietary TH challenge of 12 ppm for both T_4 and T_3 allowed the intestine's role in regulating TH through TH metabolism pathways to be compared for each TH.

Effects of dietary T_4 and T_3 challenges on hepatic TH metabolism

In liver, T_3 treatment decreased T_4ORD, increased T_4IRD, and increased T_3IRD. T_4 treatment did not affect TH metabolism pathways (Figure 4-9). All pathways in the liver required DTT as a cofactor.

T_4ORD was the main metabolism pathway affected by dietary TH challenges. With T_3 treatment, plasma T_3 levels were elevated. MacLatchy and Eales (1993) have shown that the liver is sensitive to circulating levels of T_3. With enough biologically active T_3 available in circulation, the liver decreases deiodination pathways converting T_4 to T_3. Hence in the present experiment, the liver decreased T_4ORD by Day 7. A dietary T_4 challenge did not affect T_4ORD, showing that T_3, not T_4, influences peripheral regulation of T_3 availability through modulation of peripheral deiodination of T_4 to T_3.

In addition to reducing production of biologically active TH, this experiment showed that elevated plasma T_3 increased liver T_4IRD activity. With T_3 treatment, plasma T_3 was elevated, and the liver did not convert more T_4 to biologically active T_3. T_4IRD removed the substrate T_4, preventing the synthesis of more T_3.

Effects of dietary T_4 and T_3 challenges on intestinal TH metabolism

All areas of the intestine differed from liver in that DTT as a cofactor was not required, except for T_4IRD and T_3IRD in some regions. In addition, T_4cORD, T_4IP, T_3IP production were greater in the intestine than in the liver. Of the regions of the intestine tested, TH metabolism pathways in the MI and the DI were the most active, but different TH metabolism pathways were active for these two sites. The MI and the DI were the main regions of the intestine that responded to dietary TH challenges by altering activity of TH metabolism pathways. The DI was the region of the intestine that exhibited the highest TH metabolism. Thus, TH metabolism pathways in the MI and the DI are discussed further.

Middle intestine

By Day 7, the MI responded to a dietary T_3 challenge by increasing T_4cORD, T_4IRD, T_4IP, and T_3ORD pathways (Figure 4-10). Only T_4IRD required DTT.

All of the TH metabolism pathways in the MI increased by T_3 treatment were "inhibitory" or deactivating in that T_3 was not formed as a result of increased activity of the pathways. Thus, the MI responded to a dietary T_3 challenge by removing the T_4 substrate, through T_4cORD, T_4IRD, and T_4IP pathways, or by removing the active hormone T_3, through T_3ORD pathways. Except in the cases of T_4IRD and T_3ORD, the pathways did not form another iodothyronine which could have biological properties in

the intestine. Thus, the MI was sensitive to dietary T_3 levels, and it prevented the formation of new T_3.

Distal intestine

The DI increased T_4ORD in response to a dietary T_4 challenge. The DI increased T_4ORD, and T_3IRD in response to a dietary T_3 challenge (Figure 4-11). Only T_3IRD required DTT.

The main pathways active in the DI, regardless of treatment, were T_4cORD and T_4IRD. This and other active TH metabolism pathways deiodinated both T_4 and T_3. The DI TH metabolism pathways degraded the T_4 substrate, perhaps to salvage iodide before loss through excretion. T_4cORD and T_4IRD may explain why less T_4 than T_3 appeared in the plasma, if the hormone was degraded before it could enter circulation. With T_3 treatment, T_4ORD and T_3IRD increased. T_4ORD was also induced with T_4 treatment. This pathway would convert T_4 to active T_3. However, with T_3IRD also active, the T_3 produced would be deiodinated. Thus, the process overall would inactivate and degrade TH.

Initially, TH metabolism was greatest in DI, but mainly in the controls. Since this was before treatment began and the fish were acclimated for 2 weeks, all fish should be homogenous, but control fish had higher TH metabolism. It appears that increased TH metabolism in the intestine at Day 0 represented an acute response to initial sampling. Control fish were sampled first. Thus, the stress response of elevated TH metabolism was not evident in subsequent sampling with other groups on Day 0 or on other sampling days. The fish adjusted to the disturbance, and elevated TH metabolism was not observed in controls after Day 0. This suggested that acute responses of TH metabolism pathways

in the intestine can be mediated through a stress response. Another stressor, the physical disturbance of transporting fish, is known to temporarily depress thyroidal status in rainbow trout (Johnston *et al.*, 1996). The stressor decreased hepatic production of biologically active T_3 by decreasing T_4ORD activity. At the same time, hepatic degradation of T_4 and T_3 was increased by increasing both T_4IRD and T_3IRD activity.

For all regions of the intestine under normal conditions, the overall level of TH metabolism was lower than the liver, since T_4ORD activity in the liver was much more active than in the intestine. Unlike the liver, many different TH metabolisms pathways were active in the intestine, most of which would degrade T_4 and T_3. With dietary TH challenges, the activities of the degradatory pathways were increased. The only other study on the effects of a dietary T_3 challenge on intestinal metabolism in fish was conducted on sturgeon (Plohman *et al.*, 2002). In the sturgeon, feeding 12 ppm T_3 did not induce T_3 metabolism, and neither hepatic nor intestinal T_3IRD pathways were increased.

Conclusions

The intestine, specifically the MI and DI, moderated influxes of TH. It responded to increased dietary T_3 by degrading T_3 or preventing the formation of new T_3, especially in the MI. The DI prevented the increase of T_4 into circulation, despite the high level of this hormone in the diet. The DI, in addition to responding to the presence of T_4, responded to an increase in dietary T_3 by degrading the hormones, possibly to salvage I^- before loss through excretion.

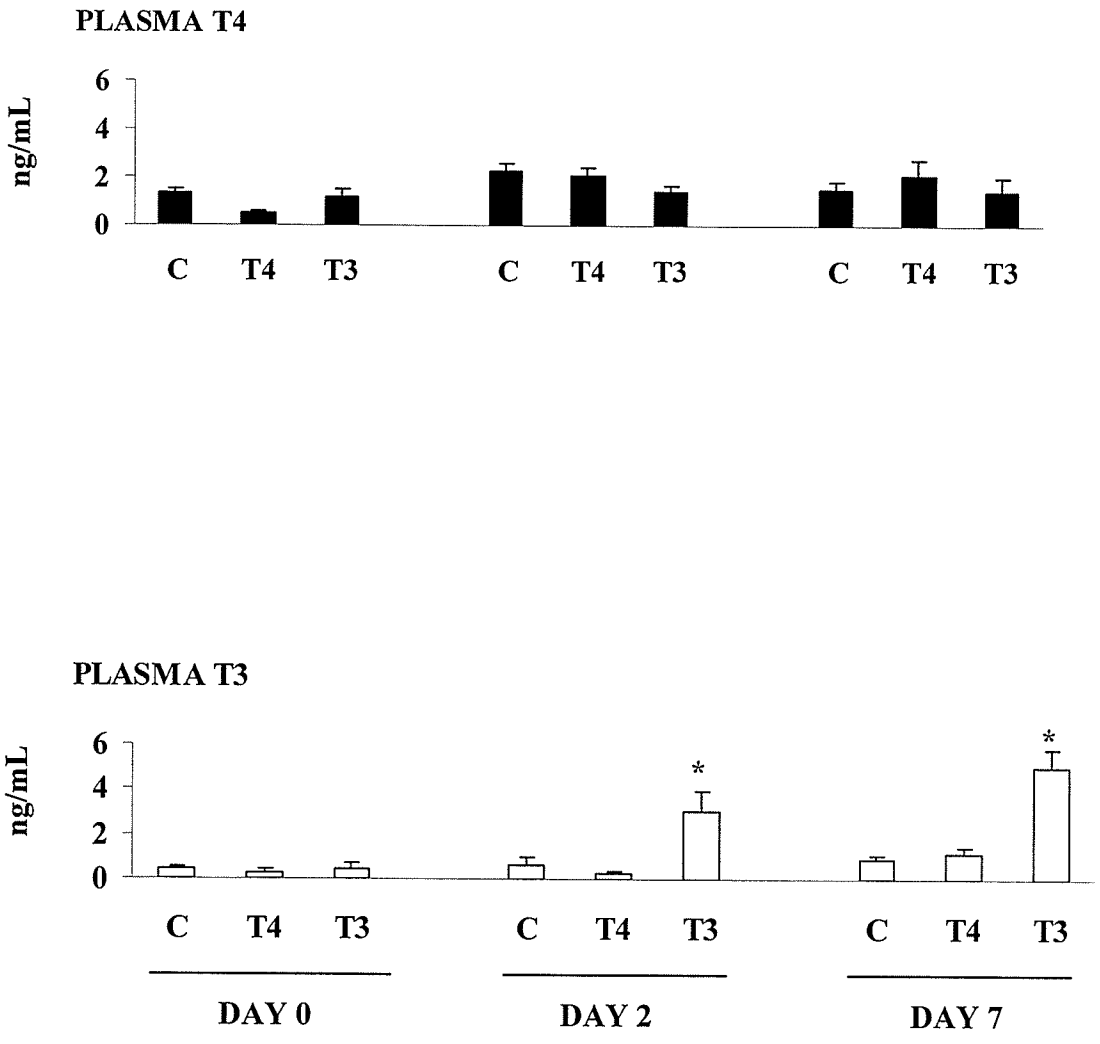


Figure 4-1. Plasma T_4 (■) and T_3 (□) levels for control (C, n=9), T_4 treatment (T_4, n=9), and T_3 treatment (T_3, n=9) over 0, 2, and 7 days of dietary TH challenges. Bars represent means (± s.e.). Asterisks indicate significant differences at $p \leq 0.05$ in T_4 or T_3 levels between treated and control fish at that day.

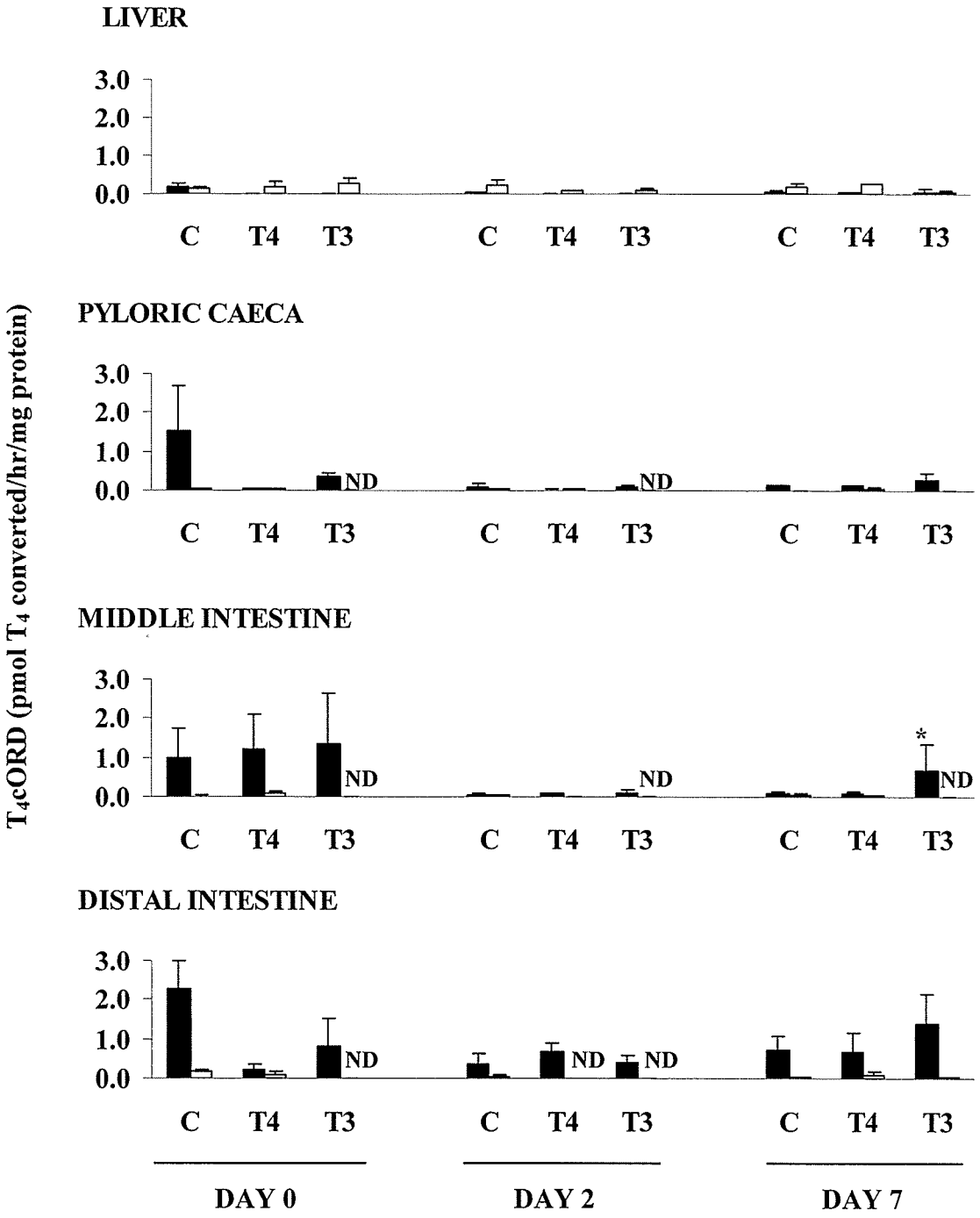


Figure 4-2. Rate of microsomal T_4cORD activity for control (C), T_4 treatment (T_4), and T_3 treatment (T_3) over 0, 2, and 7 days of dietary TH challenge. Activity was determined by HPLC separation of *T_4 metabolism products after incubation with liver (n=3-9), pyloric caeca (n=3-9), middle intestine (n=3-9), and distal intestine (n=3-9) at DTT concentrations of 0 mM (■) or 10 mM (□). Bars represent means (± s.e.). Asterisks indicate significant differences at $p \leq 0.05$ in activity between treated and control fish at that day. ND denotes non-detectable.

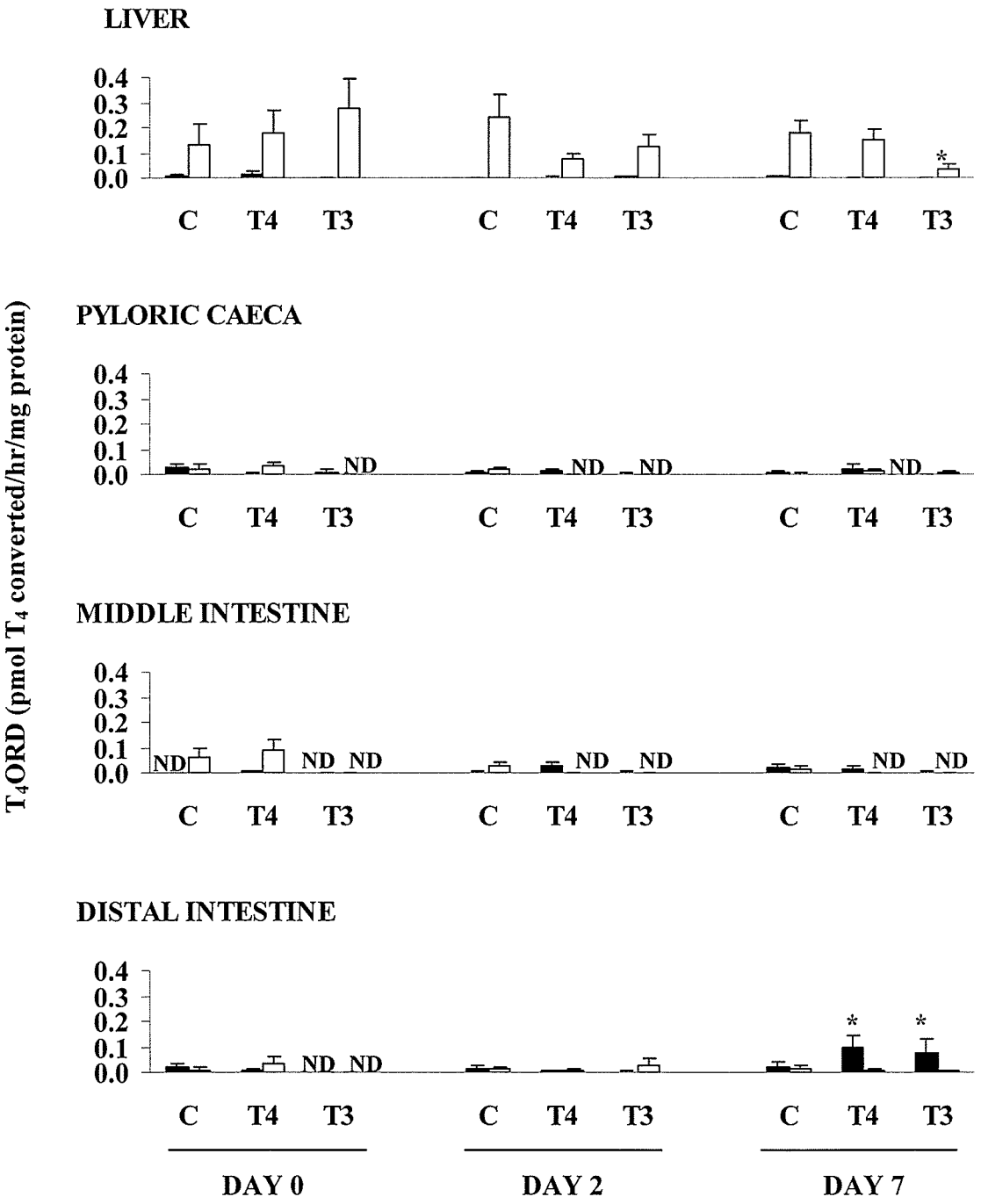


Figure 4-3. Rate of microsomal T_4ORD activity for control (C), T_4 treatment (T_4), and T_3 treatment (T_3) over 0, 2, and 7 days of dietary TH challenges. Activity was determined by HPLC separation of *T_4 metabolism products after incubation with liver (n=3-9), pyloric caeca (n=3-9), middle intestine (n=3-9), and distal intestine (n=3-9) at DTT concentrations of 0 mM (■) or 10 mM (□). Bars represent means (± s.e.). Asterisks indicate significant differences at $p \leq 0.05$ in activity between treated and control fish at that day. ND denotes non-detectable.

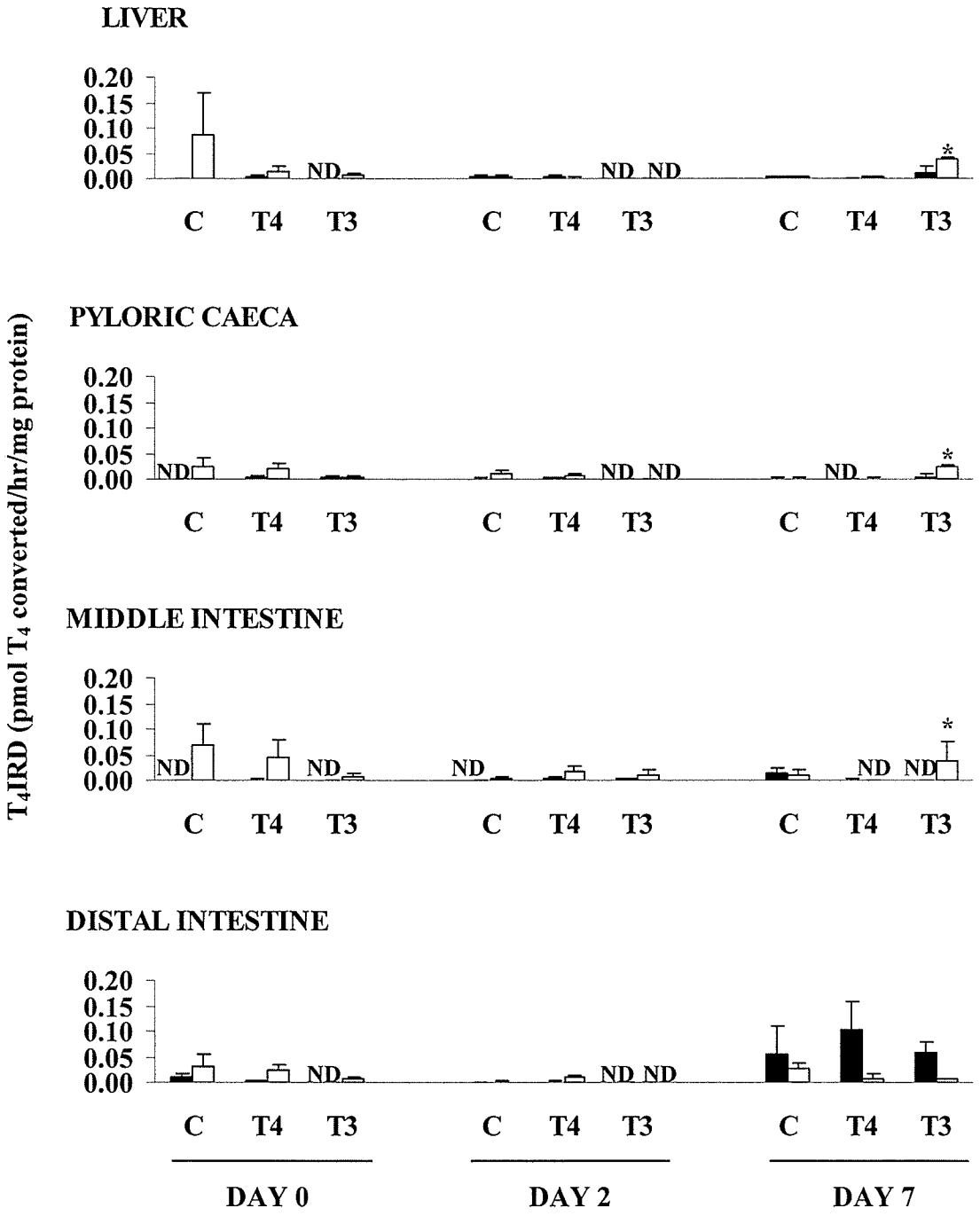


Figure 4-4. Rate of microsomal T_4IRD activity for control (C), T_4 treatment (T_4), and T_3 treatment (T_3) over 0, 2, and 7 days of dietary TH challenges. Activity was determined by HPLC separation of *T_4 metabolism products after incubation with liver (n=3-9), pyloric caeca (n=3-9), middle intestine (n=3-9), and distal intestine (n=3-9) at DTT concentrations of 0 mM (■) or 10 mM (□). Bars represent means (± s.e.). Asterisks indicate significant differences at $p \leq 0.05$ in activity between treated and control fish at that day. ND denotes non-detectable.

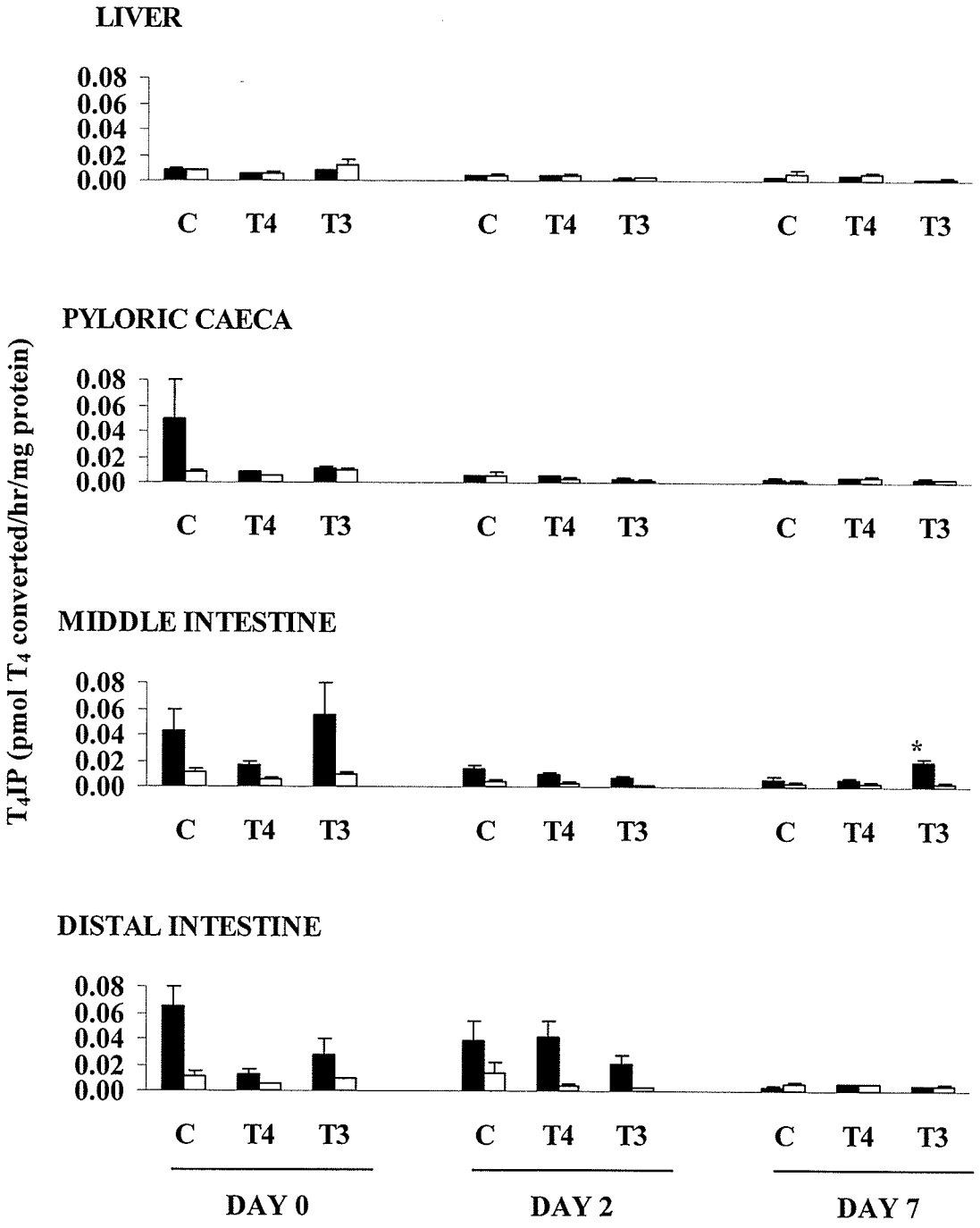


Figure 4-5. Rate of microsomal T_4IP production for control (C), T_4 treatment (T_4), and T_3 treatment (T_3) over 0, 2, and 7 days of dietary TH challenges. Activity was determined by HPLC separation of *T_4 metabolism products after incubation with liver (n=3-9), pyloric caeca (n=3-9), middle intestine (n=3-9), and distal intestine (n=3-9) at DTT concentrations of 0 mM (■) or 10 mM (□). Bars represent means (± s.e.). Asterisks indicate significant differences at $p \leq 0.05$ in activity between treated and control fish at that day.

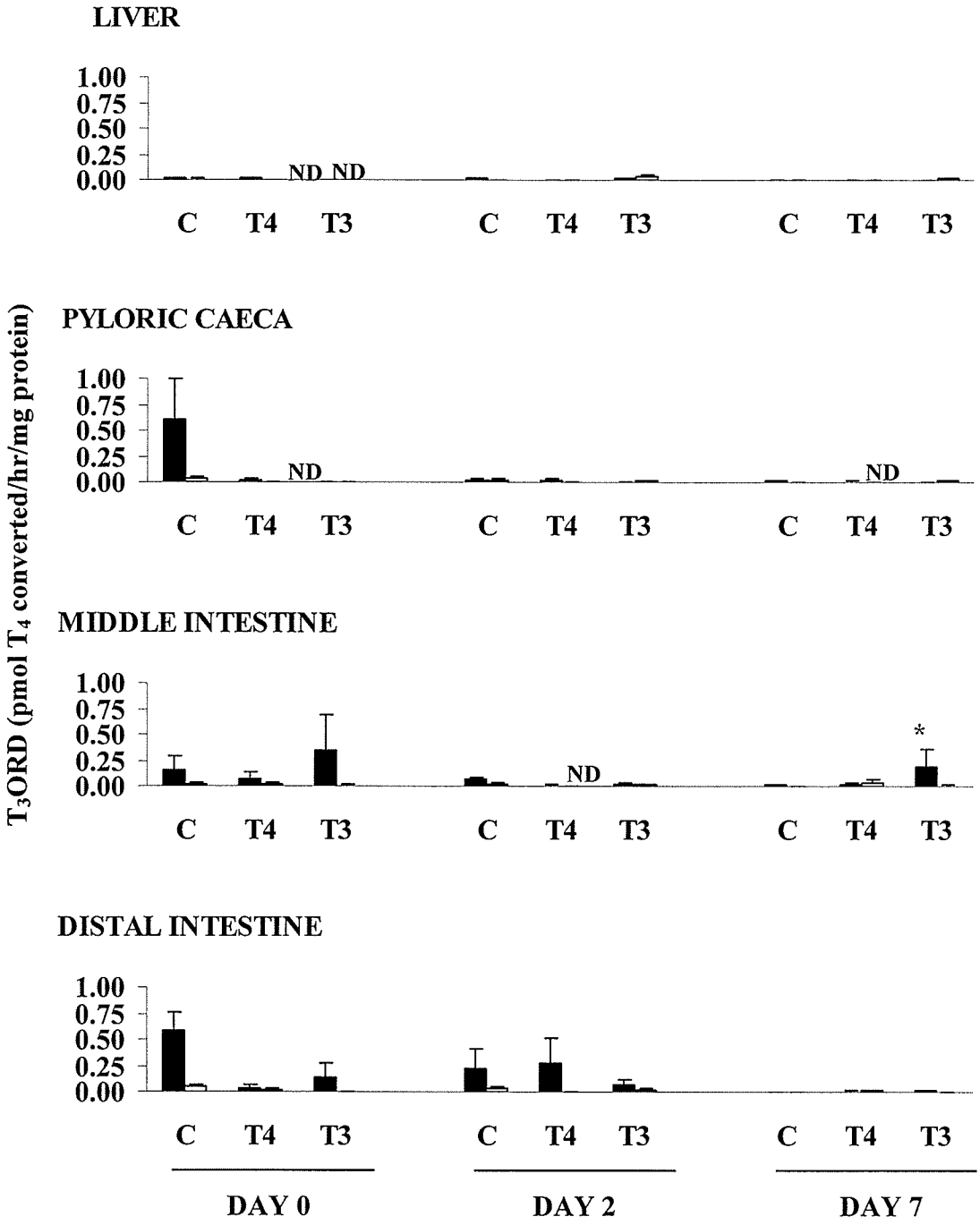


Figure 4-6. Rate of microsomal T_3ORD activity for control (C), T_4 treatment (T_4), and T_3 treatment (T_3) over 0, 2, and 7 days of dietary TH challenges. Activity was determined by HPLC separation of *T_3 metabolism products after incubation with liver (n=3-9), pyloric caeca (n=3-9), middle intestine (n=3-9), and distal intestine (n=3-9) at DTT concentrations of 0 mM (■) or 10 mM (□). Bars represent means (± s.e.). Asterisks indicate significant differences at $p \leq 0.05$ in activity between treated and control fish at that day. ND denotes non-detectable.

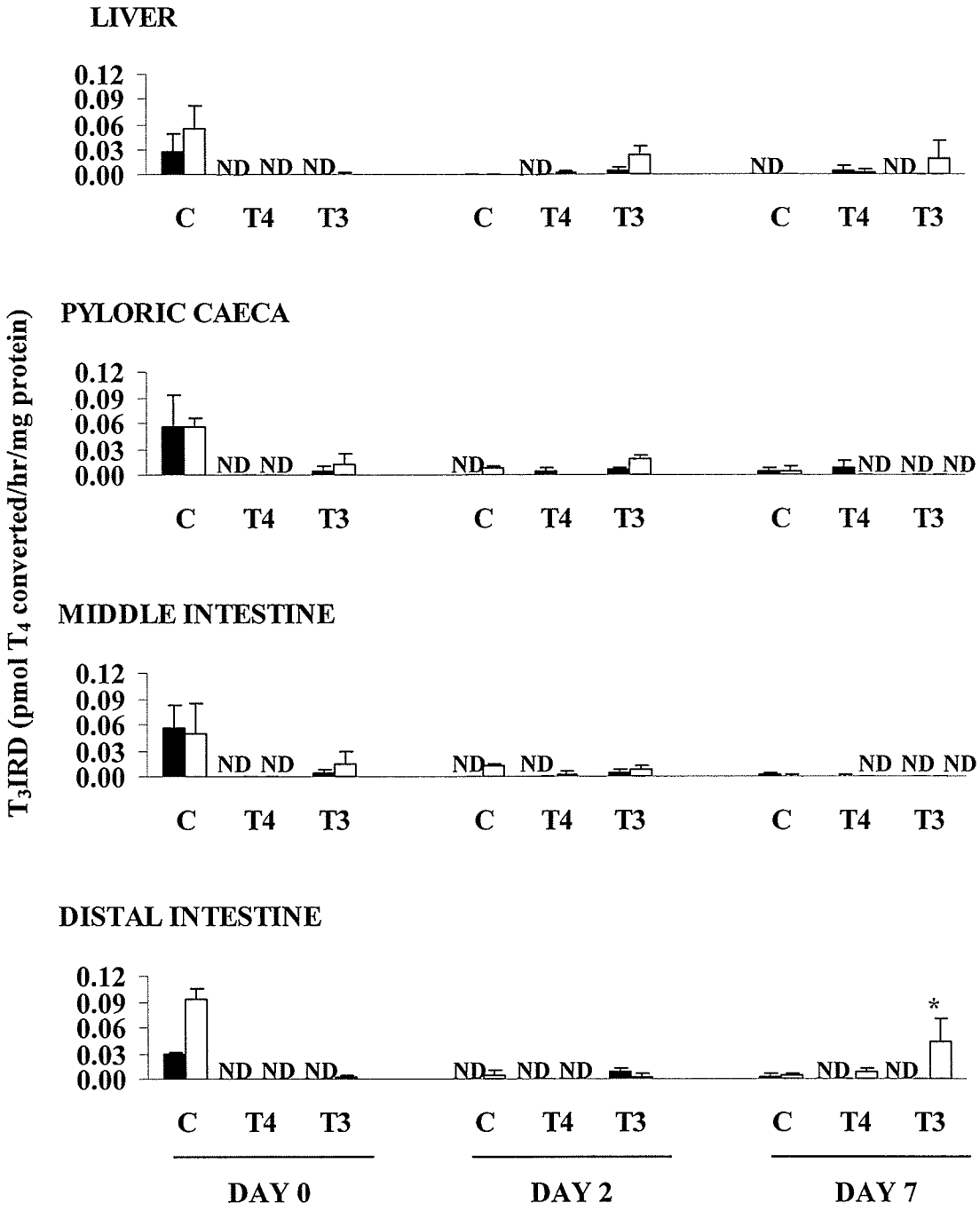


Figure 4-7. Rate of microsomal T_3IRD activity for control (C), T_4 treatment (T_4), and T_3 treatment (T_3) over 0, 2, and 7 days of dietary TH challenges. Activity was determined by HPLC separation of *T_3 metabolism products after incubation with liver (n=3-9), pyloric caeca (n=3-9), middle intestine (n=3-9), and distal intestine (n=3-9) at DTT concentrations of 0 mM (■) or 10 mM (□). Bars represent means (± s.e.). Asterisks indicate significant differences at $p \leq 0.05$ in activity between treated and control fish at that day. ND denotes non-detectable.

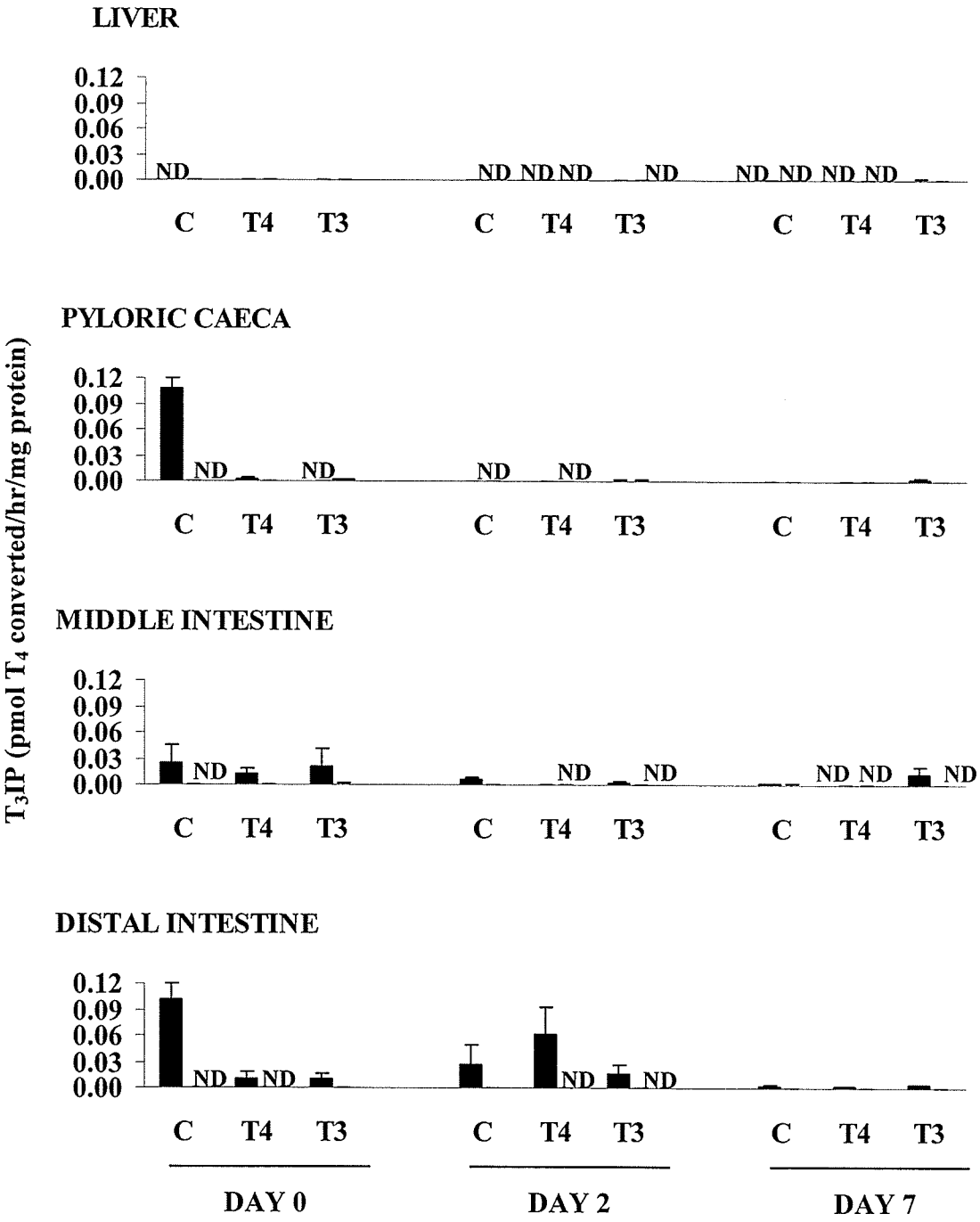


Figure 4-8. Rate of microsomal T_3IP production for control (C), T_4 treatment (T_4), and T_3 treatment (T_3) over 0, 2, and 7 days of dietary TH challenges. Activity was determined by HPLC separation of $*T_3$ metabolism products after incubation with liver (n=3-9), pyloric caeca (n=3-9), middle intestine (n=3-9), and distal intestine (n=3-9) at DTT concentrations of 0 mM (■) or 10 mM (□). Bars represent means (± s.e.). ND denotes non-detectable.

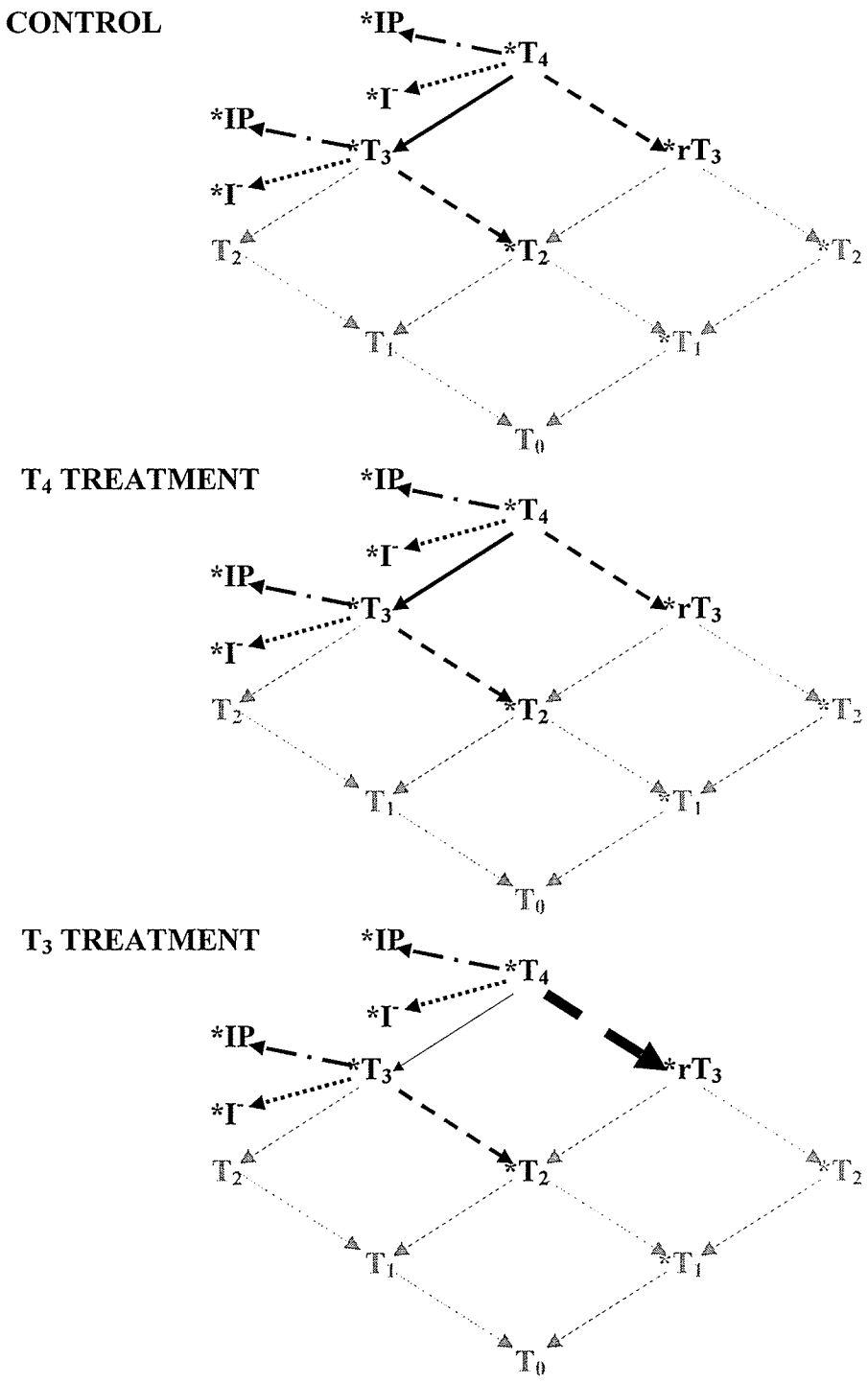


Figure 4-9. Summary of TH metabolism pathways in the trout liver. For control fish, pathways that were active are designated by black arrows. For T_4-treated and T_3-treated fish, pathways that were increased significantly are designated by thick black arrows, and pathways that were decreased significantly are designated by thin black arrows. Pathways that were not measured are designated by thin gray arrows.

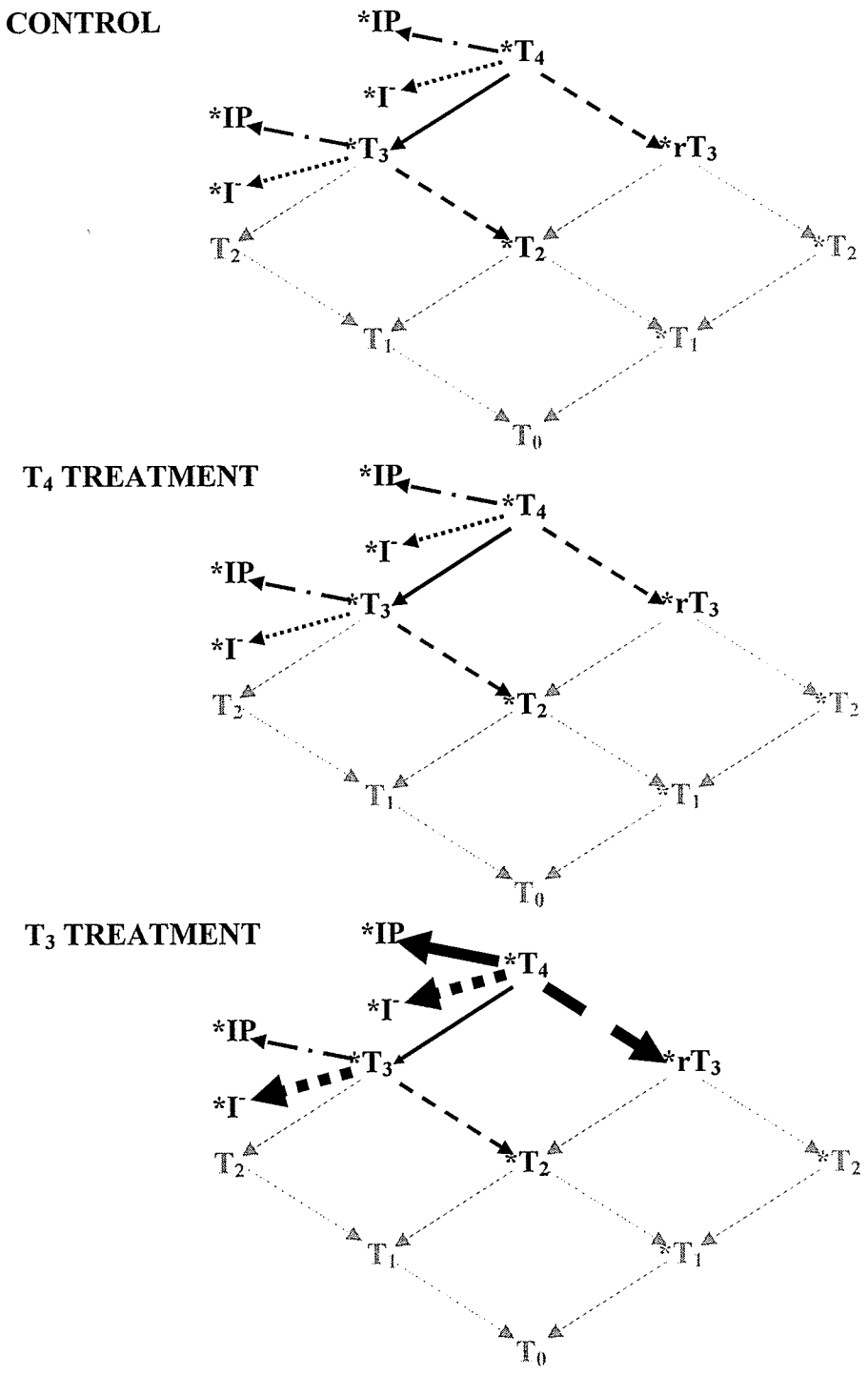


Figure 4-10. Summary of TH metabolism pathways in the trout MI. For control fish, pathways that were active are designated by black arrows. For T_4-treated and T_3-treated fish, pathways that were increased significantly are designated by thick black arrows. Pathways that were not measured are designated by thin gray arrows.

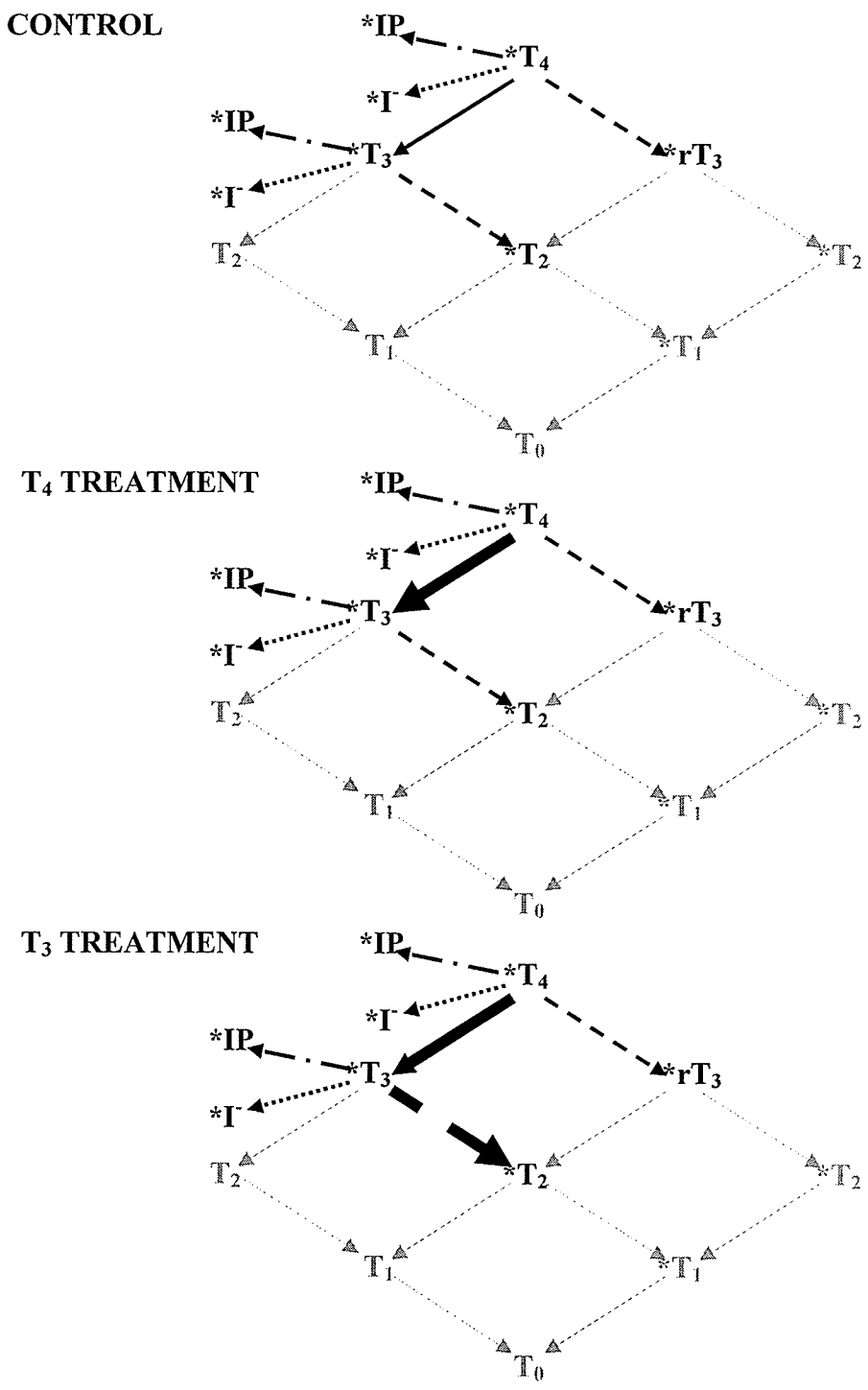


Figure 4-11. Summary of TH metabolism pathways in the trout DI. For control fish, pathways that were active are designated by black arrows. For T_4-treated and T_3-treated fish, pathways that were increased significantly are designated by thick black arrows. Pathways that were not measured are designated by thin gray arrows.

Chapter 5

Development of an *In vivo* Method to Assess the Acute Uptake of Thyroxine and Triiodothyronine by Enterohepatic Tissues of Rainbow Trout

Introduction

Enterohepatic circulation (EHC) involves the cycling of substances from the blood to the liver to the bile to the intestine and back to the blood (Figure 1-4). With EHC, the intestine is not considered solely an excretory route, rather it can also provide a dynamic pool of materials from which metabolism, temporary storage, and absorption take place. Thyroid hormones (TH), thyroxine (T_4) and triiodothyronine (T_3), can follow an EHC, depending on the form of iodothyronine released in bile and the extent of its absorption from the intestine. In mammals, biliary-excreted TH can contribute to the intestinal pool of TH (DiStefano, 1988; DiStefano *et al.*, 1991). From the intestine, unconjugated TH can be absorbed extensively and re-enter the enteric circulation (Briggs *et al.*, 1953; Chung and van Middlesworth, 1964; Sternlicht *et al.*, 1984; Hazenburg *et al.*, 1988; DiStefano, 1988; DiStefano and Harris, 1988; Rutgers *et al.*, 1989; DiStefano *et al.*, 1992). Thus, a TH EHC is created, which may contribute to the regulation of whole-body TH (DiStefano *et al.*, 1993). The same could be true for teleost fish.

There is limited knowledge of TH EHC in teleost fish. Previous studies have investigated individual components of TH EHC, and hence the potential for completion of the EHC, by evaluating intestinal access to TH through biliary supply and by evaluating reentry of TH to plasma through absorption of TH from the intestine. Results reported in Chapter 4 demonstrated the potential of the trout intestine for regulating

metabolic conversion of excess TH. The fish intestine may therefore contribute to the regulation of thyroidal status if TH discharged from bile or obtained through the diet is absorbed from the intestine and contributes to available systemic TH.

In salmonids and other teleost fish, there is abundant evidence for TH uptake by the liver and TH excretion into the bile. After an intraperitoneal injection of $*T_4$ into fasted brook trout, over 50% of the radioactivity accumulated in the bile was radiolabeled $*T_4$ ($*T_4$) (Sinclair and Eales, 1972). After an intraperitoneal $*T_4$ injection into rainbow trout, $*T_4$ and its less absorbable conjugates were also accumulated in the bile after 24 h. After a similar injection of $*T_3$, mainly $*T_3$ conjugates, rather than $*T_3$, were accumulated in bile of rainbow trout (Finnson and Eales, 1996). Furthermore, feeding brook trout caused the rapid release of bile into the intestine (Eales and Sinclair, 1974). Thus in salmonid fish studied to date, the biliary route represents a source of TH released to the intestine, with more T_4 than T_3 delivered in its native, unconjugated form.

There is far less information for teleost fish on the completion of EHC with TH uptake from the intestine. Indirect evidence of intestinal absorption of TH has been documented in rainbow trout by measuring increased levels of plasma TH after ingestion of TH-supplemented diets (Higgs *et al.*, 1979; Higgs *et al.*, 1982; Cyr and Eales, 1990; Eales *et al.*, 1990; Eales and Finnson, 1991; Sweeting and Eales, 1992; Fines *et al.*, 1999). There was consistently a greater increase in plasma T_3 than T_4 with the same dietary TH dose.

More direct measurements of TH uptake from the intestine have been made by following radiolabeled TH (*TH) injected into the lumen of a fish intestine. In starved channel catfish, 20-25% of the radioactivity associated with $*T_4$ or $*T_4$ and its

glucuronidated form (*T_4-G) were absorbed after 24 h from the lumen of a ligatured middle intestine (Collicutt and Eales, 1974). In brook trout, between 12 and 36% of the radioactivity associated with *T_4 or both *T_4 and *T_4-G injected into a double-ligatured intestinal loop of middle intestine and distal intestine was lost from the intestinal lumen after 24 h (Eales and Sinclair, 1974). Since the bile is released to the intestine only upon feeding, an experiment compared starved rainbow trout injected with *T_4 and then fed to starved controls injected with *T_4 but not fed. In the fed trout, *T_4 and *T_4-G accumulated in the bile were released to the intestine, but there was no increase in plasma *T_4 relative to unfed controls (Eales and Sinclair, 1974). This suggested that negligible cycling of TH occurred, since little TH or TH conjugates were absorbed from the intestine in comparison to the amount returning to the intestine. Thus in teleost fish, the intestine is traditionally considered an excretory pathway for TH. Though, considering its mass of tissue, the intestine cannot be ignored as a possible large pool of TH for absorption to the circulation and EHC.

Based on the above, and since a given level of dietary T_3 increased circulating T_3 levels to a greater degree than dietary T_4 increased circulating T_4 levels (Higgs *et al.*, 1979, 1982), it was believed that T_4 was more poorly absorbed than T_3 from the digestive tract. A study by Whitaker and Eales (1993) was conducted to compare T_4 and T_3 absorption from the intestinal lumen of rainbow trout. Large doses of *T_4 or *T_3 were injected into the middle intestine, via an anal catheter, and uptake was measured over a period of 48 h. In agreement with the above studies, T_3 was removed from the intestine and entered blood and tissues more extensively than T_4.

In summary, there is considerable evidence that high proportions of TH are secreted in fish bile. It has been concluded that significant biliary excretion of unconjugated TH occurs, mainly in the form of T_4. There is far less information on what appears to be limited uptake from the intestine. However, the existing data suggest that, at least in salmonid fish, a higher proportion of T_3 than T_4 can enter the circulation from the intestinal lumen. At the moment, TH uptake from the intestine to blood, liver and bile has not been addressed in a systemic manner to compare T_4 and T_3, to identify the region of the intestine involved in absorption of TH, or to describe the mechanisms involved in TH uptake.

The initial objectives of this chapter were to develop an *in vivo* method to describe and quantify the uptake (net loss) of TH from the intestinal lumen and their distribution into systemic blood, liver, and gall bladder of rainbow trout and to determine if TH absorbed from the intestine could complete an enterohepatic cycle. To achieve these various objectives, labeled TH, accompanied by either a low or a high dose of the corresponding unlabeled TH, were injected into a ligatured intestinal section in anesthetized trout, and the luminal and tissue radioactivities were determined one hour later. For each tissue, the form of radioactivity (*TH or *TH metabolites) was identified so that the distribution could be followed to a terminal point in the EHC, the gall bladder.

The intent was then to use this procedure to compare the net loss of the two primary TH, T_4 and T_3, at TH levels that could be encountered normally in the diet. The net loss of TH was used to determine if a saturable intestinal transport system might be involved in their uptake, and to determine for different regions of the intestine if there were differences between T_4 and T_3 net loss from the lumen, uptake to the liver and the

blood, and accumulation in the bile. Other variables that might affect the results were considered. Fundamental to interpretation was the determination of possible degradation of TH in the intestinal lumen prior to TH absorption. Also of interest was the effect of pre-experimental fasting. This chapter, therefore, includes data that address the following hypotheses:

1. Insignificant degradation of $*T_4$ or $*T_3$ occurs during a one-hour residence in the intestinal lumen.
2. Extended pre-experimental fasting reduces T_3 uptake from the intestinal lumen.
3. T_3 net loss from the intestine is greater than T_4 net loss.
4. The net loss of the radioactive portion of a TH injectate will be reduced in the presence of a high concentration of unlabeled TH in the lumen, demonstrating the involvement of a saturable intestinal transporter.
5. TH net loss from the intestine and uptake into the liver, blood, and gall bladder is greater in ligatured sections that include the distal intestine as opposed to those that include the middle intestine alone.

Materials and Methods

Fish maintenance

Rainbow trout were obtained from the Rockwood Hatchery (Gunton, Manitoba) and held at the University of Manitoba in running, aerated, dechlorinated, Winnipeg city water at 12°C and 12L:12D photoperiod in 60-cm x 60-cm x 60-cm tanks. They were fed once daily with commercial trout food (3.2 mm, Martin Feed Mills) at 1% of body mass.

Preparation of intestinal sections

Food was withheld routinely for three days before injection to allow the intestine to empty. However, in the experiment to test the effects of duration of fasting, food was withheld for up to 14 days prior to injection.

To double-ligature the intestine, fish were anesthetized with tricaine methanesulfonate (MS222, 0.034 g/L, Syndel Labs) and placed on their back in holders with dechlorinated water (12°C) and MS222 (0.034 g/L) pumped over the gills with a Little Giant Pump, Fisher (Figure 5-1). An incision was made on the ventral side beginning anterior to the pectoral fins, cutting laterally around the fins (to avoid severing the abdominal vein), finishing adjacent to the anus, and exposing the length of the intestine. A section of the intestine was ligatured with suture thread in two places. Care was taken to minimize disruption of blood vessels in this region.

In order to compare intestinal regions, two different sections of the intestine, MI section and MI-DI section were double-ligatured. For the MI section, the upper ligature was just posterior to the pyloric caeca and the lower ligature was just anterior to the beginning of the distal intestine at the pyloric sphincter (trout mean weight 898.1; range 660-1290 g). For the MI-DI loop, the upper ligature was just posterior to the pyloric caeca and the lower ligature was just anterior to the anus (trout mean weight 572.4; range 295-1064 g). The middle intestine was included with the distal section to ensure adequate blood supply and to simulate the site of entry if TH were in the biliary discharge.

***In vivo* injection of labeled substances into double-ligatured MI or MI-DI sections**

Intestinal injectate composed of radioactive TH (*T_4 or *T_3, >800 μCi/μg New England Nuclear) followed immediately by a non-radioactive TH dose (T_4 or T_3) was consistently injected into the MI lumen of both MI and MI-DI sections. Purified *T_4 (*I^- contamination 7.8%) or *T_3 (*I^- contamination 5.0%) was reconstituted in 0.1 N NaOH to provide approximately 4,000,000 cpm/mL. Non-radioactive TH doses (T_4 or T_3) at pre-determined molar concentrations of 200 fmoles (low dose) or 100 pmoles (high dose) were prepared in osmotically balanced Tris-HEPES buffer (0.50 g Tris, 1.39 g HEPES, and 3.64 g mannitol per 1 L H_2O; pH 7.4). 50 μL of either *T_4 or *T_3 was injected into the double-ligatured section, followed immediately by 50 μL of either T_4 or T_3 dose, to make a total of 100 μL of intestinal injectate.

To test the mechanism of TH uptake for saturability, the effects of the low TH dose of 200 fmoles and a high TH dose of 100 pmoles were compared for net loss of T_4 or T_3. The lower concentration of TH was at a level that the fish could normally be encountered in diets (MacKenzie *et al.*, 1993). The net loss of TH were measured for both MI and MI-DI sections.

Sampling

One hour after injection into the intestinal section, the already anesthetized fish were killed by a blow to the head, and the cardiac whole blood, liver, bile, and intestinal section (tissue and contents) were collected and weighed. Sampling was done at one hour to provide sufficient time for measurable uptake while minimizing the possibility of TH metabolism in the intestinal tissue. For all tissues, except for bile, 2 mL of a protease

(0.025 g/mL of buffer, Pronase, Sigma) was added, and radioactivity was measured in the gamma counter against the injected dose.

Tissue extractions

After 24 h of protease digestion at 25°C, 2 mL of methanolic ammonia ($MeOH:NH_4OH$; 99:1; v/v) containing methylmercaptoimidazole (MMI, 0.115 g/mL) as a reducing agent was added to all but bile, vortexed, and centrifuged at 1420 g for 20 min. The supernatant was saved, and extraction of the pellet with $MeOH:NH_4OH$ was repeated two more times. The pooled supernatants for individual tissue extracts were dried at 37°C under an airstream.

Identification of radioactive substances

The evaporated tissue extracts were reconstituted in 0.05 N NaOH and added to LH-20 Sephadex mini-columns (5 mL Quick Sep Column, Isolab), each containing 0.25 g of LH-20 Sephadex and equilibrated with 750 µL of 0.1 N HCl. Bile was added directly to the LH-20 column. The TH metabolites (such as *TH conjugates and $*I^-$) were eluted with 3 mL of H_2O, and then the total TH fraction (*TH) was eluted with 3 mL of ethanolic ammonia ($EtOH:NH_4OH$, 99:1, v/v). The fractions were counted in the gamma counter.

Column recovery of TH for each tissue was tested by adding a known amount of $*T_4$ or $*T_3$ to a tissue (approximately 5,000 cpm), digesting with protease, extracting with $MeOH:NH_4OH$, and separating the *TH metabolism products and *TH on LH-20 columns. By measuring both the amount of TH recovered from $*T_4$ or $*T_3$ alone and the amount of TH recovered from $*T_4$ or $*T_3$ in the tissues, a correction factor for column recovery could be determined for each tissue processed (Appendices VII and VIII).

Calculations

Several calculations were made to evaluate the EHC of T_4 and T_3 through determinations of loss of the injectate from the intestinal section, uptake of radioactivity to blood and liver, accumulation of radioactivity in bile, and the forms of radioactivity in these tissues. These determined whether TH could cycle to and from the intestine.

1. **Net loss of total injected radioactivity from the intestinal section (%)**

= (cpm radioactivity lost from section/cpm radioactivity injected) x 100

Initial attempts were made to separate the intestinal contents from the intestinal tissue itself. However, washing the intestinal tissue with ethanol to remove residual luminal contents may have extracted intracellular hormone from the intestinal tissue or removed the mucosal layer. Thus, the percent of the total injected radioactivity removed from the entire intestinal section (tissue and contents) was used to determine loss of the radioactive injectate. This calculation represented the percent of total radioactivity (*TH or *TH metabolites) absorbed from the intestinal section that could potentially enter the EHC one hour after injection of $*T_4$ or $*T_3$.

2. **Percent *TH in the injectate, intestinal section, liver, blood, or bile (%)**

= (cpm *TH from the column fraction/cpm total tissue radioactivity)/100

The fraction of total radioactivity represented by *TH was determined for the injectate, intestinal section, liver, blood, or bile, respectively. This was determined by separating the *TH from possible *TH metabolites (such as *TH conjugates and $*I^-$) on LH-20 mini-columns. Thus, *TH in the injectate or tissues was calculated as the percent of the total radioactivity in the tissue that was eluted as *TH.

The percent *TH in the injectate and intestinal sections was used to determine metabolic breakdown of *TH and *I⁻ contamination after processing samples, thus evaluating the degradation of *TH during a one-hour residence in the intestinal lumen.

The percent *TH was also used in later calculations to determine *TH uptake by the liver or blood and to determine accumulation of *TH in the bile.

3. Uptake by the liver or blood

a. Total radioactivity uptake by the liver or blood (%/mg)

= [(cpm tissue/cpm injectate) x 100/mg tissue] x (body weight/500)

Uptake of total radioactivity into the liver or the blood was calculated as the percent of the total radioactive injectate taken up by a milligram of tissue, and this was then corrected for isotope dilution due to body weight by normalizing to a 500-g fish. This calculation was used to further calculate *TH uptake by the liver or blood and *TH metabolite uptake by the liver or blood.

b. *TH uptake by the liver or blood (%/mg)

= (Total radioactivity uptake into tissue) x (Percent *TH in tissue/100)

By using calculations 2 and 3a, the amount of total radioactivity taken up by the liver or blood was adjusted to represent only the amount of radioactivity remaining as *TH. *TH uptake to the liver represented the amount of TH from the intestine entering the liver, a main peripheral regulator of available systemic TH and the source of biliary TH. *TH uptake to the blood represented the amount of TH the intestine could make available to the tissues through systemic circulation.

c. *TH metabolite uptake by the liver or blood (%/mg)

= (Total radioactivity uptake into tissue) – (*TH uptake into tissue)

*TH uptake by the liver or blood (calculation 3b) was subtracted from the total radioactivity uptake by the liver or blood (calculation 3a). This represented the radioactivity in the tissue that was no longer in the form of the *TH injected, rather it was *TH metabolites such as *TH conjugates and *I⁻.

4. Accumulation in the bile

a. Total radioactivity accumulation in the bile (%)

= (cpm total radioactivity in bile/cpm injectate) x 100

Net accumulation of total radioactivity in the bile was determined as a percent of injected dose. The bile represented the contents of a terminal sac that could be emptied into the intestine. It would contain the stored *TH and *TH metabolites that would be released to the intestine upon feeding. Thus, neither the amount of bile nor the isotope dilution due to body size were factors in calculating uptake, only the amount of radioactivity accumulated. This calculation was further used to calculate accumulation of *TH in the bile and accumulation of *TH metabolites in the bile.

b. *TH accumulation in the bile (%)

= (Accumulation total radioactivity in bile) x (Percent *TH bile/100)

From calculations 2 and 4a, accumulation of unconjugated *TH in the bile was determined. This calculated the portion of the radioactivity in the bile that remained in the form of the *TH injected, potentially returning to the intestine in the EHC.

c. *TH metabolite accumulation in the bile (%)

= (Accumulation total radioactivity in bile) – (Accumulation of *TH in bile)

The accumulation of *TH in the bile was subtracted from the accumulation of total radioactivity in the bile. This represented the radioactivity in the bile that was no longer in the form of the *TH injected, rather it was *TH metabolites such as *TH conjugates and *I⁻.

Protocol

The above parameters were measured under various conditions to address previously stated hypotheses:

1. Degradation of TH during one-hour residence in the intestinal lumen

The proportion of *TH in the injectate standard was compared to the proportion of *TH remaining in the injected intestinal section after an hour. A significant difference between these values would indicate that TH metabolism occurred in the intestinal lumen.

2. Length of fasting prior to injection

Because intestinal digesta can bind TH and restrict their reabsorption, and because this variable was difficult to control, all studies were conducted on fish fasted at least 3 days, which is the time required to clear the distal intestine. This lead to a preliminary study to determine if extended fasting (3-5 days, 6-10 days, 11-14 days) prior to injection of T_3 (low dose of 200 fmoles) reduced T_3 net loss from the intestinal lumen. T_3 was chosen as the test hormone, since previous studies (see Introduction) suggested that T_3 was the main TH likely to be absorbed from the intestinal lumen.

3. Form of TH injected

The net loss of T_3 from the intestinal section was compared to net loss of T_4. In all cases, the injection of a consistent dose of *TH (*T_4 or *T_3) was followed immediately by an injection of the corresponding non-radioactive TH.

4. Dose of TH injected

The uptake of the *TH injected was compared in the presence of a low (200 fmoles) or a high (100 pmoles) level of unlabeled TH in the lumen in order to determine involvement of a saturable intestinal transporter.

5. Region of intestine used to form the double-ligatured intestinal section

The middle region of the intestine alone (MI section) and the middle and distal regions of the intestine together (MI-DI section) were compared for differences in net loss of TH from the intestinal lumen, uptake by the liver or blood, and accumulation in the bile. The injection of *TH was consistently into the middle section (site of bile discharge), downstream from the upper ligature. The lower ligature was tied either at the junction of the middle and distal intestine to create the MI section, or close to the anus to create the MI-DI section. The sites for ligatures were chosen to simulate biliary discharge with the injection site, and to ensure adequate blood supply to and from the intestinal sections. In addition, TH metabolism occurred in the middle and distal intestine, with greater activity in the distal region (Chapters 3 and 4).

Statistics

Using the SPSS statistics package, univariate analysis of variance (ANOVA) was used to compare means of either net loss of total radioactivity from the intestine, total uptake by the liver, total uptake by the blood, or total accumulation in the bile for effects

of the length of pre-experimental fasting. Univariate ANOVA was used to compare means for net loss of TH from the intestinal section for effects of TH injectate (T_4 low dose, T_4 high dose, T_3 low dose, and T_3 high dose). Each test was performed individually for MI or MI-DI sections. These tests were followed by LSD post hoc multiple comparisons to determine significant differences among TH injectates. For each analyzed (either net loss of TH from the intestinal section, TH uptake by the liver, TH uptake by the blood, or TH accumulation in the bile), independent t-tests were used to compare means between MI section and MI-DI section. Each test was performed individually for TH injectate doses. Statistics were not performed on percent *TH (that was used to determine degradation of TH during one-hour residence in the intestinal section) due to low and variable sample sizes. Results were determined statistically significant at $p \leq 0.05$.

Results

Degradation of TH during one-hour residence in the intestinal lumen

The comparisons of percent *TH in the injectate vs. percent *TH in the intestinal section were made several days after processing, and some non-biological degradation of *TH would be expected (Table 5-1). Thus, the percent *TH in the injectate was lower than when measured at injection (> 92% TH with less than 8% $*I^-$ contamination). However, it was assumed that non-biological degradation would occur at the same rate in injectate and samples.

In all instances, the percent *TH in the standard injectate fell within the range of variation of the percent *TH in the intestinal section, indicating that negligible intestinal

TH metabolism occurred after one hour. Thus, it was assumed that net loss of radioactivity from the intestinal section represented absorption of the intact *TH injected.

Length of fasting prior to injection of $*T_3$

Fasting before injection of $*T_3$ (low dose) did not significantly affect net loss of injectate from the MI-DI section ($F=0.100$, d.f.=2, $p=0.909$), total uptake to the blood ($F=2.405$, d.f.=2, $p=0.206$), total uptake to the liver ($F=2.588$, d.f.=2 $p=0.169$), or total accumulation of injectate in the bile ($F=0.7488$, d.f.=2, $p=0.496$; Table 5-2).

Form of TH injected

For the MI section, there was a significant difference in net loss among injectates ($F=7.200$, d.f.=2, $p=0.034$) such that net loss of T_3 high dose from the intestine was significantly greater than net loss of T_4 high dose (Table 5-3). The greatest net loss from the intestine was T_3 high dose, followed by T_4 high dose, and then T_3 low dose.

For the MI-DI section, there was no significant difference in net loss among injectates ($F=1.399$, d.f.=2, $p=0.342$). Though, net loss of T_4 high dose (17%) was greater than net loss of T_3 at either high (6.1%) or low (6.0%) doses. Thus, in the MI section, T_3 was absorbed more than T_4, but in the MI-DI section, T_4 was absorbed more than T_3.

Dose of T_3 injected

For the MI section, net loss of T_3 from the intestine increased significantly with an increase in unlabeled TH injected ($F=7.200$, d.f.=2, $p=0.013$; Table 5-3).

For the MI-DI section, net loss of T_3 low dose was comparable to that for the T_3 high dose, and there was no change in net loss of T_3 with an increase in unlabeled T_3

injected. Thus, for both sections, there was no decrease in net loss of T_3 from the intestinal lumen with the use of a potentially saturating dose of unlabeled T_3.

Region of intestine used to form the double-ligatured intestinal section

Fate of T_4 injected into intestinal sections

The fate of the T_4 low dose was examined for the MI-DI section only (Table 5-4). Its net loss was not measured. The liver had a significant level of *TH metabolites (11.1%/mg), and it appeared that some of these entered the systemic circulation. The majority of the injectate present in the systemic circulation was in the form of *TH metabolites (0.0315%/g). From the liver, 0.0013% of the total injectate was accumulated as *TH in the bile, however, the majority of the radioactivity was in the form of *TH metabolites (0.0087%).

For the MI section, the net loss of T_4 high dose was 10.7%. The intestine's contribution to *TH available to tissues (via liver) was mainly in the form of *TH metabolites (0.0133%/g). *TH uptake by the liver was comparable to the amount of *TH metabolite uptake by the liver. Little of the *TH injected remained as *TH when it was taken up by the systemic blood; most of it was in the form of *TH metabolites. No *TH was accumulated in the bile, even though 0.0028% of the total injectate was accumulated in the bile as *TH metabolites.

Net loss of T_4 high dose from the MI-DI section was 17.3%. The majority of the injectate taken up into the liver and the blood was in the form of *TH metabolites. However, only *TH was accumulated in the bile (0.0210%). Overall, injection of T_4 into the MI-DI section, rather than the MI section alone, resulted in significantly greater TH uptake by the liver ($t=3.323$, d.f.=9, $p=0.010$), uptake by the blood ($t=2.237$, d.f.=9,

p=0.054), but not significantly greater net loss from the intestine (t=0.685, d.f.=3, p=0.542) or accumulation in the bile (t=0.886, d.f.=14, p=0.087).

Fate of T_3 injected into intestinal sections

Net loss of T_3 low dose from the MI section was 5.4% (Table 5-5). All of the injectate taken up by the liver remained in the form of *TH. This was also true for uptake by the systemic circulation. The form of radioactivity accumulated in the bile was not assayed.

Net loss of T_3 low dose from MI-DI section was 6.1%. This was not significantly different than net loss of T_3 low dose from the MI (t=0.317, d.f.=2, p=0.782). Not all of the original injectate remained in the form of *TH. This contrasts with results from the MI section. The majority of the injectate taken up by the liver remained in the form of *TH, and this amount was significantly greater than for the MI section (t=2.789, d.f.=4, p=0.049). However, the majority of the injectate entering the systemic circulation was in the form of *TH metabolites. Thus, the amount of *TH in the blood was not significantly different than the amount of *TH in the blood for the MI section (t=2.239, d.f.=4, p=0.089). The form of radioactivity accumulated in the bile was unknown, but the total radioactivity accumulated in the bile was greater than for the MI section. Thus, net loss of T_3 low dose was similar for both intestinal sections, however, greater amounts of *TH circulated from the intestine to tissues in EHC after absorption from the MI-DI section in comparison to the MI section.

Net loss of T_3 high dose from the MI section was 15.4%. Despite this high net loss, none of the radioactivity in the systemic circulation remained in the form of *TH. The form of radioactivity in the liver was not assayed. From the liver, no *TH was

accumulated in the bile, but 0.0073% of the total injectate was accumulated in the bile as *TH metabolites.

Net loss of T_3 high dose from the MI-DI section was 6.0%. The levels of *TH and *TH metabolites present in the liver were comparable, yet the majority of the injectate from the intestine entering systemic circulation via the liver remained as *TH. Uptake to blood was greater for the MI-DI section. Only *TH was accumulated in bile. Even though net loss of T_3 high dose from the MI-DI section was significantly less than from the MI section ($t=3.644$, d.f.=4, $p=0.022$)), a greater amount of T_3 from the MI-DI section entered tissues in EHC and was accumulated in the bile ($t=1.806$, d.f.=6, $p=0.012$), allowing for greater *TH return to the intestine.

Discussion

In rainbow trout, there are no previous studies on the role of specific regions of the intestine in acute, *in vivo* EHC of T_4 or T_3. The present goal was to establish an *in vivo* technique to measure net loss of T_4 and T_3 from double-ligatured intestinal sections, their uptake to blood and liver, and their accumulation in bile in anesthetized, fasted trout. Had the trout been fed, the release of bile back to the intestine would have completed the cycle. Based on the results obtained, the hypotheses presented in the Introduction are addressed here in the Discussion.

1. Insignificant degradation of T_4 or T_3 occurs during a one-hour residence in the intestinal lumen

Insignificant degradation of T_4 or T_3 occurred during the one-hour residence in the intestinal lumen of the MI section or the MI-DI section. In addition, comparable percent

TH found in the injectate standard and the injected intestinal section indicated that *TH metabolites (most likely *I⁻) were not preferentially absorbed over *TH from either intestinal section, thus not altering the percent *TH in the intestinal section.

This information was fundamental to the interpretation of the data. In the study by Whitaker and Eales (1993), *T_3 injected into the MI remained in its parent form after 24 hours. After *T_4 injection, however, radioactivity in the lumen was predominantly *I⁻, and the majority of the radioactivity absorbed to plasma was also *I⁻. Since experiments described in Chapters 3 and 4 demonstrated the ability of the intestine, especially the MI and DI, to metabolize TH after one hour, the concern was that significant loss of TH through degradation would be interpreted as net loss of TH through absorption. Also a concern was that *I⁻ contamination could mask the uptake of *TH. When measuring net loss, uptake, and accumulation of the injectate, it was unknown whether *I⁻ entered from contamination of the injectate, TH metabolism in the intestine, or TH metabolism in other tissues, and each source of *I⁻ would require a separate adjustment. Hence, a correction was not feasible. However, insignificant degradation of *TH occurred, so it was assumed that net loss from the intestinal section represented TH absorption.

2. Extended pre-experimental fasting did not reduce T_3 uptake from the intestinal lumen.

Duration of pre-experimental fasting did not significantly affect net loss of T_3 from the MI-DI section, total radioactivity uptake to blood and liver, or accumulation of total radioactivity in bile. Even though it appeared that T_3 net loss increased with pre-experimental fasting, the changes were not significant, probably due to low sample size and animal variation. However, subsequent studies were performed consistently within

3-5 days of fasting, which provided enough time for intestinal contents to be cleared. In mammals, intestinal contents were shown to reduce absorption of TH from the intestine (Cottle, 1964; Chung and van Middlesworth, 1967; Ruegamer *et al.*, 1967; Heroux and Petrovic, 1969). However, prolonged fasting in fish changed intestinal intracellular structure (McLeese and Bergeron, 1990) decreased microvillar length (Hossain and Dutta, 1991; McLeese and Bergeron, 1990), altered enzymatic activities (Mommsen *et al.*, 2003) and decreased absorption of nutrients such as amino acids (Collie, 1985; Avella *et al.*, 1992). Prolonged fasting can also alter physiological state, such as thyroidal status, in rainbow trout. A decrease in nutrient intake leads to inhibition of thyroidal function and peripheral production of T_3 (Eales, 1988; Leatherland, 1994). This could alter the intestine's response to luminal TH. With short-term fasting of 3-5 days, influences of both digesta binding the injectate and confounding effects of nutritional status on intestine were minimized. In addition, radioactivity from all tissues was separated using columns to determine the form of radioactivity counted.

Even though short-term fasting may have altered the physiological state of the fish, all studies were conducted under the same conditions. The TH that was delivered to the bile from the liver was not released to the intestine, and this was advantageous. The amount of *TH and *TH metabolites that would cycle to the intestine could be collected. They did not confound the calculations of original injectate net loss and distribution to EHC tissues.

3. T_3 net loss from the intestine is greater than T_4 net loss.

For the MI section, greatest net loss from the intestine was measured for the T_3 high dose, followed by the T_4 high dose, and then the T_3 low dose. This only partially

supports previous data suggesting that T_3 is more readily absorbed than T_4. Furthermore, for the MI-DI section the greatest net loss from intestine was measured for the T_4 high dose.

Previous data supporting greater T_3 absorption than T_4 were based on feeding TH in the diet and then measuring physiological markers such as growth (Higgs *et al.*, 1979) or measuring plasma TH levels (Sweeting and Eales, 1992b). However, these indirect measurements of TH absorption may instead reflect physiological mechanisms other than intestinal absorption that regulate plasma TH levels, since this study indicated that both TH were absorbed from the intestine, with T_4 absorbed to a greater extent than T_3 from the MI-DI section.

The entry of an "excess" of TH into circulation may induce mechanisms to maintain TH homeostasis despite TH uptake from the intestine. For T_4, regulation by feedback at the level of the hypothalamus and pituitary could reduce T_4 production from the thyroid. For both T_4 and T_3, peripheral TH metabolism such as deiodination and conjugation could maintain static plasma TH levels.

Plasma T_4 levels may be affected by negative feedback at the hypothalamic-pituitary level so that T_4 absorbed from the intestine is offset by reduced thyroidal T_4 secretion. T_4, which is the main hormone released by the thyroid, exerts a strong negative feedback on the hypothalamo-pituitary-thyroid axis, but T_3 exerts little influence at this level (Eales and Himick, 1991). Thus, a decrease in thyroidal production of T_4, in conjunction with liver metabolism and accumulation in the bile, may effectively reduce circulating T_4 levels as a result of T_4 absorption from the intestine.

As shown earlier in this chapter, the data on percent TH in the injectate standard vs. the injected intestinal section supported the view that the form of TH injected was the same as that absorbed from the intestine. This was not the case in a previous study in which the TH injected resided for 24 hours in the intestine (Whitaker and Eales, 1993). In that study, the $*T_4$ injected into the MI was mainly in the form of $*I^-$ after 24 hours. It is possible that other studies that support greater T_3 absorption than T_4 were biased by a greater propensity for T_4 metabolism in the intestine than T_3.

For the MI-DI section in particular, greater absorption of T_4 than T_3 may not be evident by measuring plasma levels, especially in experiments extending longer than one hour. In the present study, TH injected at the site of bile entry were able to move to the DI, and a small proportion was absorbed within one hour. Higher doses of T_4 (and T_3) in the MI-DI section resulted in unmetabolized *TH accumulated in the bile.

The liver is the site where TH are discharged to the gall bladder. An increase in TH can lead to greater biliary excretion. For both $*T_4$ and $*T_3$ injected into the MI section at a high dose, the liver accumulated *TH metabolites in the bile. However, T_4 was not preferentially metabolized over T_3, suggesting that peripheral metabolism did not reduce plasma T_4 to a greater extent than T_3.

For experiments of longer duration, T_4 may not be available for absorption, since it can be degraded in the intestinal tissue. If it does get absorbed from the intestine, it can be accumulated in the bile. Finally, if it enters systemic circulation, it can inhibit thyroidal secretion of T_4.

4. Net loss of the radioactive portion of T_3 injectate was not reduced in the presence of a high concentration of unlabeled TH in the lumen, not supporting the involvement of a saturable intestinal transporter.

After *T_3 injection, *TH net loss was not reduced by a 500-fold increase of unlabeled TH. This suggests that a saturable TH transporter is not involved. However, a T_3 carrier system that is very abundant or saturable at a higher dose cannot be excluded. The difference in net loss of T_3 at low and high doses for the MI section and the MI-DI section suggest that there are regional differences in the mechanism of absorption of T_3. The transport systems for absorption of TH from the intestine have not been studied in any animal, to date. However in fish, the transport of TH into hepatocytes and red blood cells (RBC) have been characterized for rainbow trout.

In the liver, various T_4 and T_3 uptake and inhibition properties suggested that T_4 and T_3 were transported to a small extent by diffusion, but that T_4 and T_3 had separate binding sites for transport (Riley and Eales, 1994). Yet, both transporters shared characteristics in that transport into hepatocytes was an energy-dependent, Na^+-independent, carrier-mediated and possibly an endocytotic process. Transport was not inhibited by tyrosine and phenylalanine. Thus, TH were not transported into hepatocytes by the same system used by structurally similar aromatic amino acids.

Transport of TH into RBC differed from hepatocyte uptake. T_4 and T_3 were taken up into RBC by both nonsaturable and saturable systems (McLeese and Eales, 1996a, 1996b). In addition to simple diffusion, separate transport systems were characterized for T_4 and T_3. Rapid T_3 transport was energy-independent and Na^+-independent, which

resembled the T system for aromatic amino acid transport. Slower T_4 uptake did not involve the amino acid transporter.

All tissues may not necessarily have the same mechanism for TH transport across cell membranes. Different tissues appear to have different TH uptake mechanisms, most likely based on their varying roles in regulating thyroidal status. For example, separate hepatic T_4 and T_3 uptake may allow independent regulation of peripheral metabolism. Separate, rapid uptake of T_3 by RBC may allow stable levels of plasma T_3 for autoregulation. In future studies, uptake by enterocytes should be characterized to determine differences in T_4 and T_3 transport properties and to determine regional differences in the intestine. Energy requirements, Na^+ requirements, and inhibition by amino acid concentrations should be tested. This would elucidate whether aromatic amino acid transporters were used for TH uptake or if separate transporters have evolved for intestinal TH absorption. Since uptake of various nutrients is regionally distributed, the differential uptake of TH may be determined by different transporters that are regionally distributed.

5. TH net loss from the intestine and uptake by the liver, blood, and gall bladder is greater in ligatured sections that include the distal intestine as opposed to those that include the middle intestine alone.

Overall, the enterohepatic parameters of TH net loss from the intestine and uptake into the liver, blood and gall bladder were greater in ligatured sections that included the distal intestine as opposed to those that included the middle intestine alone. These parameters were evaluated together to determine the potential for TH EHC and what tissues were involved in regulating the potential TH EHC. The intestine and the liver are

the main enterohepatic tissues with the ability to regulate the amount of TH cycling. The intestine serves as a source of TH entering the EHC. From intestinal saturation studies with T_3, the intestine does not appear to regulate acute doses of TH entering the EHC, since an increased TH dose injected into the lumen results in increased net loss from the intestinal loop. The liver is the pivotal tissue that determines what returns to the intestine via biliary route. It has the ability to regulate the amount and form of TH entering the bile, since it is the main peripheral tissue involved in TH metabolism through deiodination or conjugation. Thus, the liver may regulate the amount of TH cycling.

MI section

The MI section did not appear to contribute to T_4 or T_3 EHC. For T_4, *TH were taken up by the liver, however little *T_4 was measured in the blood and no *T_4 was measured in the bile. Thus, T_4 could not return to the intestine upon refeeding, completing the T_4 EHC. Radioactivity accumulated in the bile was represented by *T_4 metabolites such as *TH conjugates or *I⁻. Conjugates are more water soluble (Visser, 1990) and do not appear to lead to deiodination in trout (Finnson and Eales, 1996), representing an excreted form of TH. The biliary route was an excretory pathway for a high dose of T_4 which was peripherally metabolized, maintaining low levels of T_4 in systemic circulation.

T_3 EHC was negligible or non-existent for the MI section. T_3 was taken up by the blood at low dose, maintaining circulating T_3 available to systemic tissues. However, it was not determined what percent of the radioactivity accumulated in the bile was *T_3. Even if only T_3 was accumulated in the bile, the amount of radioactive injectate potentially returning to the intestine was minimal. For the T_3 high dose, no T_3 uptake was

measured in the blood, and no *T_3, only *T_3 metabolites, were accumulated in the bile. Thus T_3 could not cycle to and from the MI section. With an increase in T_3, peripheral tissues quickly metabolized and cleared *T_3 via the biliary route, preventing a systemic increase in biologically active T_3.

MI-DI section

In the MI-DI section, EHC of T_4 was most likely to occur, since net loss from the intestine, TH uptake to the liver and the blood, and accumulation in the bile were the greatest. At a low T_4 dose, little *T_4, mainly *T_4 metabolites, were accumulated in the bile after peripheral metabolism. Thus, at the low levels of T_4, little *T_4 remained in circulation, and it would return to the intestine as *T_4 metabolites. At the high levels of T_4, absorption from the MI-DI section increased, and only unmetabolized *T_4 was accumulated in the bile. Together, these indicated an increased potential for T_4 EHC at a high dose of T_4 injected. As with the high T_4 dose, the liver responded to a high dose of T_3 by accumulating only *T_3 in the bile. Thus, the potential for T_3 EHC also existed, but it was less than for T_4.

For both T_4 and T_3, an acute increase in intestinal injectate dose altered TH cycling for the MI-DI section. At high doses, the amount of TH accumulated in the bile was 100% TH. This was different from the metabolism of TH seen in the MI section. The increased percent TH in bile may be a result of saturation of the enzymes in the liver that conjugate excess TH for excretion and removal from the system. Glucuronidation by uridine diphosphoglucuronosyl transferase (UDPGT) and sulfation by phenol sulfotransferases (PST) are significant TH metabolism pathways in rainbow trout, especially for T_4 (Finnson and Eales, 1996, 1997). Such conjugation modifies TH into a

more water-soluble, less absorbable form (Visser, 1990). Hence, this metabolism of TH is considered an excretory pathway. Previous experiments on various teleost fish have shown that up to 50% of TH is conjugated in the liver and excreted to the bile (Sinclair and Eales, 1972; Eales and Collicutt, 1974; Finnson and Eales, 1996). Finnson and Eales (1997) have shown that the UDPGT enzyme is sensitive to various iodothyronines. For example, T_4 can inhibit T_3 glucuronidation. Thus, an increased percent TH in the bile may be a result of saturation or inhibition of enzymes in the liver.

Whereas a greater percent *TH in the bile could be considered excretion of excess hormone through the gastrointestinal tract, this would not necessarily be a loss of TH from the system. The intestine is a large mass of tissue, and TH in bile entering the intestine could be bound by digesta, temporarily stored, and later transported back into circulation. Overall, the presence of T_4 or T_3 in the MI section resulted in absorption, degradation, and excretion of TH through the biliary pathway. This may be to prevent fluctuations of TH, especially T_3 which is the biologically active form that is more readily absorbed from this region. The presence of T_4 or T_3 in the MI-DI section resulted in greater absorption but also accumulation in the bile as the parent form of TH injected. This represented differential regulation by the liver, allowing TH EHC for TH absorbed from the distal intestine. The presence of TH in different regions of the intestine may activate control of the liver through hormones or the nervous system. This may allow salvaging of TH from the distal region before loss through excretion.

Conclusions

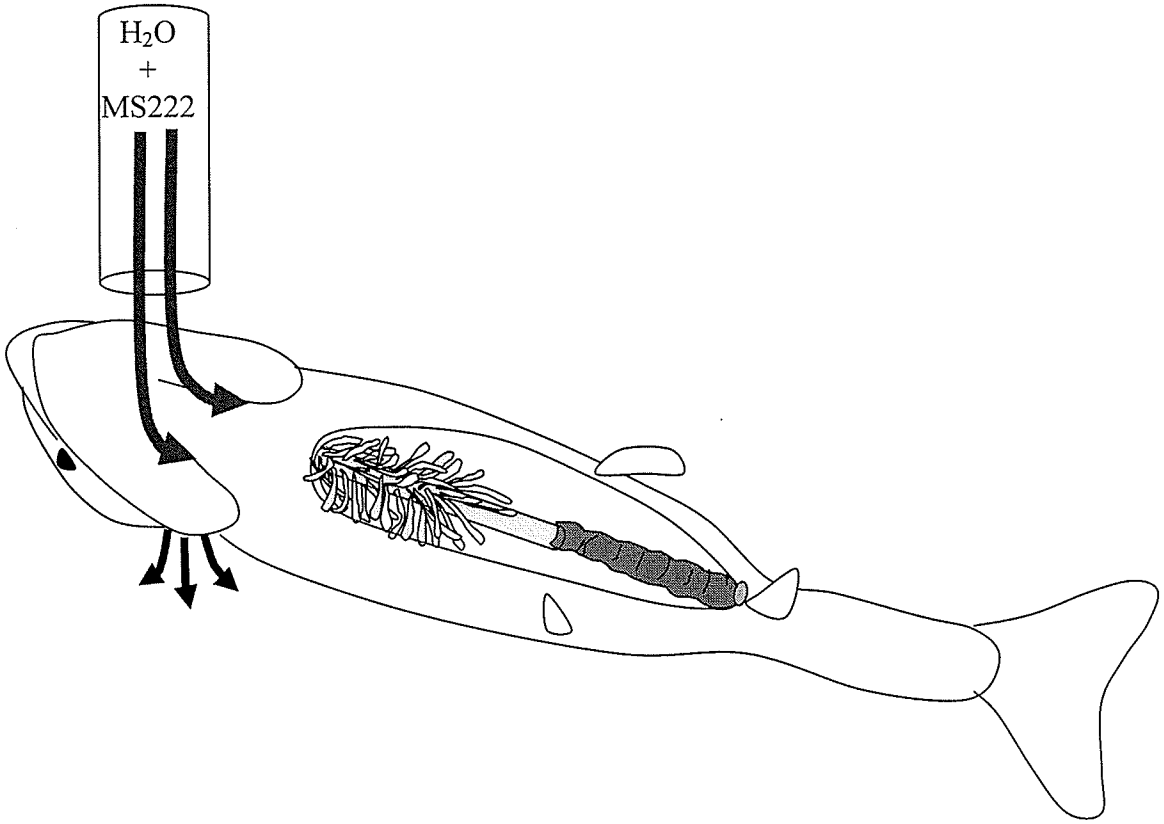
From these experiments, several specific points were concluded. In regards to measuring net loss of TH from the intestine, insignificant degradation of T_4 or T_3

occurred within a one-hour residence in the intestinal lumen of the MI or the MI-DI section. Thus, net loss from the ligatured intestinal section represented TH absorption. Another concern was that the duration of pre-experimental fasting would affect net loss from the intestine. By performing experiments within 3-5 days of withholding food, TH could not bind to digesta, and the TH cycling to and from the intestine could be measured with insignificant alterations to the physiological state of the fish. Net loss of TH from the intestine depended on the form of TH injected and the region of the intestine investigated. From the MI, T_3 was absorbed to a greater extent. T_4 was absorbed more readily from the DI. However, the duration of TH residence in the intestinal lumen would affect results, as negative feedback at the hypothalamus and pituitary, degradation, and biliary excretion can influence the availability of systemic TH. There was no evidence for saturable TH transporters for TH intestinal absorption. However, a carrier system that is very abundant or saturable at a higher dose cannot be excluded. Since absorption of TH was dependent on the form of TH and the region of the intestine, there may be different transporters for T_4 and T_3 distributed regionally.

With TH absorption from the intestine and accumulation in the bile, the potential for TH EHC was evaluated. The MI did not appear to contribute to T_4 or T_3 EHC. This may be to prevent fluctuations of TH, especially T_3 which is the biologically active form that is more readily absorbed from this region. The MI and DI together can potentially cycle TH to and from the intestine, with T_4 returning to a greater extent than T_3. This may allow salvaging of TH from the distal region before loss through excretion. Therefore the intestine, through absorption and EHC, could serve as an extrathyroidal source of TH for tissues.

This study established an *in vivo* method to evaluate the complete TH EHC from the intestine to blood to liver to bile. Using the *in vivo* method established, it was shown that the intestine served as a source of TH. However the intestine itself did not regulate increases of TH within one hour. Neither TH metabolism nor saturation at high TH doses occurred in the intestinal lumen. Rather, the liver moderated TH EHC through TH metabolism and accumulation of *TH and *TH metabolites in the bile. Previously, the intestine was shown to regulate TH metabolism under TH challenges, so the question was asked whether the intestine could regulate TH EHC with similar TH challenges. In the next chapter, the effects of chronic dietary TH challenges on potential TH EHC were examined. This was to determine if and how the availability of TH in EHC may be regulated by the intestine, hence the role of the intestine in regulating thyroidal status.

Set-up



Double-ligatured intestinal sections

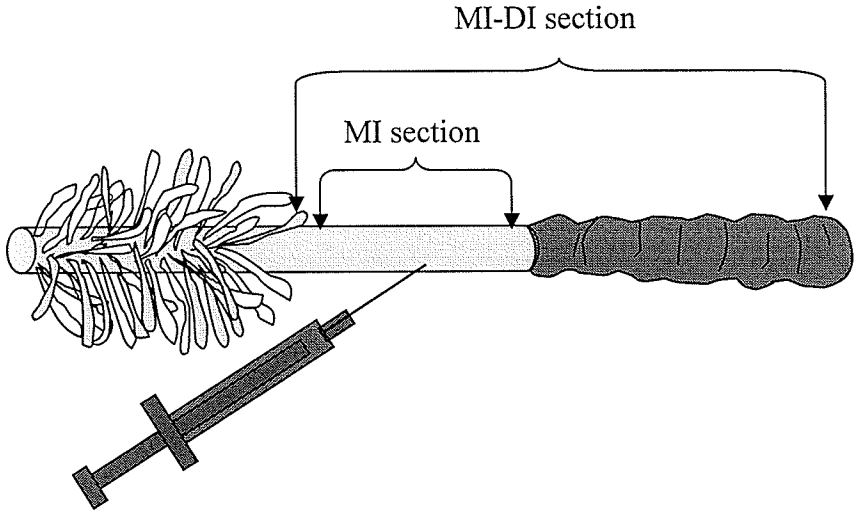


Figure 5-1. Methods used for characterization of *in vivo* absorption of TH from a double-ligatured intestinal section. The set-up demonstrates the flow of water and anesthesia over the gills while the intestine was exposed. The double-ligatured intestinal sections demonstrate where ligatures were tied to make MI sections and MI-DI sections. The site of injection in the MI, as indicated, was consistent for both sections.

Table 5-1. Comparisons of the injectate standard (STANDARD) and the injected intestinal section (INTESTINE) when analyzed for the percent of the radioactivity remaining as labeled hormone (%*TH) after one hour. Injectate was $*T_4$ or $*T_3$ with a low dose (200 fmoles) or high dose (100 pmoles) of the corresponding unlabeled TH. Intestine includes luminal contents and tissue from the double-ligatured MI or MI-DI section that was injected with *TH. N.A. denotes not assayed.

	PERCENT *TH REMAINING (%*TH)			
	T_4 LOW	**T_4 HIGH**	**T_3 LOW**	**T_3 HIGH**
MI				
STANDARD (n=1)	N.A.	72.3	77.7	65.8
INTESTINE (n=3)	N.A.	71.1 ± 11.9	77.7 ± 7.6	69.4 ± 9.3
MI-DI				
STANDARD (n=1)	56.1	77.6	93.4	78.7
INTESTINE (n=3)	69.9 ± 7.2	78.9 ± 4.1	N.A.	66.2 ± 7.1

Table 5-2. Effects of duration of pre-experimental fasting on the fate of *T_3 injected one hour previously into the lumen of the double-ligatured MI-DI section.

	LENGTH OF PRE-EXPERIMENTAL FASTING (days)		
	3-5 (n=4)	**6-10** (n=3)	**11-14** (n=3)
NET LOSS (%)*	6.1 ± 6.1	11.0 ± 5.6	13.3 ± 12.2
TOTAL UPTAKE			
LIVER (%/mg)	68.3 ± 24.3	20.7 ± 11.5	16.0 ± 8.0
BLOOD (%/mg)	23.0 ± 9.6	3.7 ± 1.9	2.5 ± 1.5
BILE (%)*	0.0670 ± 0.0195	0.0530 ± 0.0189	0.0136 ± 0.0023

* units in percent of total injectate (%),
instead of percent of total injectate/mg tissue * g of body weight/500g (%/mg)

Table 5-3. Net loss of *T_4 and *T_3 injected one hour previously into the lumen of the double-ligatured MI section and MI-DI section, accompanied by either a low dose (200 fmoles) or a high dose (100 pmoles) of the corresponding unlabeled TH. N.A. denotes not assayed. Letters indicate significant differences at $p \leq 0.050$.

	NET LOSS FROM INTESTINAL SECTION (%)			
	T_4 LOW	**T_4 HIGH** (n=9)	**T_3 LOW** (n=4)	**T_3 HIGH** (n=9)
MI[a]	N.A.	10.7 ± 2.9	5.4 ± 1.1	15.4 ± 2.2
MI-DI	N.A.	17.3 ± 6.2	6.1 ± 0	6.0 ± 1.4

[a] $p=0.034$ among injectates

Table 5-4. Fate of *T_4 injected one hour previously into the lumen of the double-ligatured MI section, accompanied by either a low dose (200 fmoles) or a high dose (100 pmoles) of the corresponding unlabeled TH. N.A. denotes not assayed. Letters indicate significant differences at $p \leq 0.050$.

	T_4 LOW		**T_4 HIGH**	
	MI	**MI-DI** (n=3)	**MI** (n=3)	**MI-DI** (n=9)
NET LOSS (%)*				
	N.A.	N.A	10.7 ± 2.9	17.3 ± 6.2
UPTAKE BY LIVER (%/mg)				
TH	N.A.	1.9 ± 0.3	5.7 ± 1.5	23.2 ± 7.7[a]
TH MET	N.A.	11.1 ± 1.7	5.3 ± 1.5	72.8 ± 24.3
UPTAKE BY BLOOD (%/mg)				
TH	N.A.	0.5 ± 0.1	0.2 ± 0.2	24.7 ± 7.3[b]
TH MET	N.A.	31.5 ± 8.9	13.3 ± 2.3	63.3 ± 18.7
ACCUMULATION IN BILE (%)*				
TH	N.A.	0.0013 ± 0.0003	0	0.0210 ± 0.0120
TH MET	N.A.	0.0087 ± 0.0037	0.0028 ± 0.0020	0

[a] p=0.010 MI vs. MI-DI

[b] p=0.054 MI vs. MI-DI

*units in percent of total injectate (%),
instead of percent total injectate/mg of tissue * g of body weight/500g (%/mg)

Table 5-5. F ate of *T_3 injected one hour previously into the lumen of the double-ligatured MI section, accompanied by either a low dose (200 fmoles) or a high dose (100 pmoles) of the corresponding unlabeled TH. N.A. denotes not assayed. Letters indicate significant differences at $p \leq 0.050$.

	T_3 LOW		**T_3 HIGH**	
	MI (n=3)	**MI-DI** (n=4)	**MI** (n=3)	**MI-DI** (n=5)
NET LOSS (%)*				
	5.4 ± 1.1	6.1 ± 0	15.4 ± 2.2	6.0 ± 1.4[a]
UPTAKE TO LIVER (%/mg)				
TOTAL†			0.0017 ± 0.0007	
TH	0.3 ± 0.3	45.1 ± 16.1[b]	N.A.	0.7 ± 0.1
TH MET	0	22.9 ± 7.9	N.A.	0.3 ± 0.9
UPTAKE TO BLOOD (%/mg)				
TH	0.3 ± 0.3	5.9 ± 2.5	0	4.7 ± 1.6[c]
TH MET	0	17.1 ± 7.5	2.7 ± 1.5	0.3 ± 0.4
ACCUMULATION IN BILE (%)*				
TOTAL†	0.0003 ± 0.0002	0.0670 ± 0.0390		
TH	N.A.	N.A.	0	0.0070 ± 0.0002[d]
TH MET	N.A.	N.A.	0.0073 ± 0.0048	0

[a] p=0.022 MI vs. MI-DI

[b] p=0.040 MI vs. MI-DI

[c] p=0.044 MI vs. MI-DI

[d] p=0.012 MI vs. MI-DI

† total radioactivity uptake or accumulation reported, when *TH uptake and *TH metabolite uptake were not assayed.

* units in percent of total injectate (%), instead of percent total injectate/mg of tissue * g of body weight/500g (%/g).

Chapter 6

Effects of Chronic Dietary Thyroid Hormone Challenges on *In vivo* Uptake of Thyroxine and Triiodothyronine by Enterohepatic Tissues of Rainbow Trout

Introduction

In mammals, the intestine provides a dynamic pool of thyroid hormones (TH), thyroxine (T_4) and triiodothyronine (T_3), where metabolism, storage, and absorption take place (DiStefano, 1988). Under induced hypothyroidism, rats excrete less T_3 and absorb more T_3 and T_4 from the intestine, compensating for low available T_3 (DiStefano *et al.*, 1993). Thus, the intestinal pool is utilized under physiological changes to maintain thyroidal status.

In rainbow trout and other teleost fish, negligible work has been done on the role of the intestine in regulating TH. In trout, peripheral tissues contribute to the regulation of thyroidal status (Eales, 1995). Hence, the intestine, with access to TH, may contribute to TH availability through enterohepatic circulation (EHC) of TH from the intestine to the blood to the liver to the bile and back to the intestine. Rainbow trout intestine has access to TH through the diet, the systemic blood circulation, intestinal metabolism, and the biliary supply (Figure 1-4). TH enter through the diet (MacKenzie *et al.*, 1993). Dietary TH challenges (Sweeting and Eales, 1992b) or injections of TH into the intestine (Whitaker and Eales, 1993) have shown that TH can be absorbed from the intestine, resulting in plasma T_3 circulating at higher levels than plasma T_4 levels. TH may be excreted into the bile as TH or TH conjugates, with T_4 conjugated to a greater extent than T_3 (Finnson and Eales, 1997). Yet, previous studies ultimately conclude that negligible

T_4 and limited T_3 recycling occurs through enterohepatic routes for channel catfish (Collicutt and Eales, 1974), brook trout (Eales and Sinclair, 1974) and rainbow trout (Whitaker and Eales, 1993).

Despite these conclusions, findings reported in Chapter 4 suggest a role of the intestine in regulating fluctuating levels of dietary TH available for absorption by modulating TH metabolism. In Chapter 5, it was shown that loss of TH from the intestinal lumen and uptake into EHC tissues could be measured one hour after injection of *TH into the lumen. These results also showed that the potential for T_4 EHC was greater than that for T_3 EHC, and that the middle and distal intestinal section (MI-DI section), not the middle intestinal section alone (MI section), were involved in TH EHC. Furthermore, acute increases in injectate dose resulted in increased potential TH EHC. Therefore, the intestine, through TH EHC, could serve as an extrathyroidal source of TH.

In this chapter, investigations of the role of the intestine in contributing to the regulation of thyroidal status following dietary T_4 or T_3 challenges are described. This was evaluated by determining the loss from the lumen and uptake into EHC tissues of labeled T_4 ($*T_4$) or labeled T_3 ($*T_3$) injected with the corresponding unlabeled TH at low (200 fmoles) or high (100 pmoles) doses into the MI-DI section after trout were fed T_4- or T_3-supplemented food for 7 days. Owing to the need to clear the intestine of digesta before these measurements could be made, all trout were fasted for 3-4 days following dietary T_4 or T_3 challenges, immediately prior to injection of *TH. Both T_4 and T_3 were used in the experiments based on the assumption that T_4 and T_3 metabolism are to a considerable degree independently regulated (Eales and Brown, 1993). Consequently, experimental conditions were designed to investigate the following hypotheses:

1. Dietary T_4 or T_3 challenges 3-4 days previously will elevate plasma T_3 but not plasma T_4 levels.
2. Net loss of T_4 from the MI-DI section is greater than net loss of T_3.
3. T_4 and T_3 uptake across the wall of the intestinal lumen do not involve saturable transport systems.
4. A prior dietary T_4 challenge, not a dietary T_3 challenge, will modify aspects of T_4 metabolism by the enterohepatic tissues.
5. A prior dietary T_3 challenge, not a dietary T_4 challenge, will modify aspects of T_3 metabolism by the enterohepatic tissues.

Materials and Methods

Fish maintenance

Rainbow trout were obtained from Rockwood Hatchery (Gunton, Manitoba) and held at the University of Manitoba in running, aerated, dechlorinated, City of Winnipeg water at 12°C and 12L:12D photoperiod. Trout (mean weight 1116.5 g; range 409-2033 g) were acclimated in 3 tanks (60-cm x 60-cm x 60-cm) for 7 days with 6 fish per tank and fed once daily with commercial trout food (3.2 mm, Martin Feed Mills) at 1% of body mass.

Experimental protocol

To address the various hypotheses stated in the introduction, three groups of trout (n=3 per group) were used to test the effects of dietary TH challenges. Following acclimation, each group was fed either trout food sprayed with T_4 dissolved in methanol to provide 12 ppm T_4 (T_4 TRT), food sprayed with T_3 dissolved in methanol to provide

12 ppm T_3 (T_3 TRT), or food sprayed with methanol alone (control). In order to generate the final sample sizes (n=3-9), this was repeated six times during August 30, 2000 to November 10, 2000. After 7 days of experimental feeding, trout were fasted for 3-4 days to allow the intestine to empty. They were then injected with intestinal perfusate consisting of $*T_4$ or $*T_3$ (>800 μCi/μg, New England Nuclear) accompanied by the corresponding unlabeled TH at a low dose (200 fmoles) or a high dose (100 pmoles) consistently injected into the lumen of the middle intestine of a double-ligatured MI-DI section. Trout were sampled one hour later.

Preparation of intestinal sections

Trout were anesthetized, the body cavity opened, and a section of the intestine that contained the middle and distal regions was double-ligatured to form the MI-DI section, as described in Chapter 5. The placement of the ligatures ensured adequate blood supply to and from the intestine by keeping large vessels intact. The intestinal section included the site of entry if TH were in biliary discharge. In addition, the MI-DI section included regions of the intestine where deiodination was known to occur, as measured in Chapters 3 and 4.

Sampling

The trout tissues (intestinal section and contents, liver, cardiac whole blood, and bile) were sampled one hour after injection into the MI-DI section, as described in Chapter 5. An additional 3 mL of blood was centrifuged at 17,000 g for 5 min to separate the plasma, with 0.5 mL frozen at -76°C for later analysis of plasma TH levels using radioimmunoassays.

Tissue extractions

For all tissues, except for bile, the radioactivity was extracted with methanolic ammonia after 24 hours of protease digestion, as described in Chapter 5.

Identification of radioactive substances

Radioactive substances were identified as *TH or *TH metabolites for tissue extracts and bile after separation on LH-20 columns, as described in Chapter 5. The correction factor for column recovery can be found in Appendices VII and VIII.

Plasma T_4 and T_3 levels

Using solid-phase radioimmunoassay (RIA) modified from Brown and Eales (1977), Omeljaniuk *et a.l* (1984) and Kohel *et al.* (2001), separate T_4 and T_3 RIA were performed on G-25 Sephadex columns, as described in Chapter 4.

Calculations

Several calculations were made to evaluate the EHC of T_4 and T_3 through determinations of loss of injectate from the intestinal section, uptake of radioactivity by the liver or blood, accumulation of radioactivity in the bile, and the forms of radioactivity in these tissues. Details on the following calculations are described in Chapter 5.

1. Net loss of total injected radioactivity from the intestinal section (%).
2. Percent *TH in the intestinal section, liver, blood, or bile (%).
3. Uptake by the liver or blood
    a. Total radioactivity uptake by the liver or blood (%/mg).
    b. *TH uptake by the liver or blood (%/mg).
    c. *TH metabolite uptake by the liver or blood (%/mg).

4. Accumulation in the bile

   a. Total radioactivity accumulation in the bile (%).

   b. *TH accumulation in the bile (%).

   c. *TH metabolite accumulation in the bile (%).

Experimental tests

The above parameters were measured under various conditions to test the previously stated hypotheses.

1. Effects of dietary T_4 and T_3 challenges on plasma TH levels.

Plasma T_4 and T_3 levels were measured to determine whether dietary TH challenges resulted in physiological increases in TH. This prediction was based on data from Chapter 4 in which trout were fed TH until one day before sampling and plasma T_3, but not plasma T_4, was significantly elevated. This was assessed by comparing plasma T_4 and T_3 levels among controls, T_4 TRT, and T_3 TRT.

2. Comparisons of T_4 and T_3 net loss from the intestinal lumen.

Net loss of T_4 from the intestinal lumen was compared to net loss of T_3. This was in part a re-examination of a hypothesis tested in Chapter 5 by repeating the techniques with, in most cases, a larger sample size, but this was further investigated by following net loss of TH from the intestine after dietary T_4 and T_3 challenges. This was accomplished by comparing net loss of T_4 and T_3 for control fish. This was repeated separately for T_4-treated fish and T_3-treated fish.

3. Effects of a high dose of T_4 or T_3 on T_4 or T_3 net loss from the intestinal lumen.

The uptake of the radioactive portion of TH injected was measured to see if there was a reduction in the presence of a high concentration of unlabeled TH in the lumen, demonstrating the involvement of a saturable intestinal transporter. To measure this, the non-radioactive dose was varied to include a low TH dose of 200 fmoles and a high TH dose of 100 pmoles. This was a re-examination of a hypothesis in Chapter 5 by repeating the techniques with larger sample sizes, but this was further investigated by measuring net loss of TH high dose from the intestine after dietary T_4 and T_3 challenges. This was accomplished by comparing T_4 low and high dose net loss for controls and by comparing T_3 low and high dose net loss for controls. This was repeated separately for T_4-treated fish and T_3-treated fish.

4. Effects of dietary T_4 or T_3 challenges on $*T_4$ metabolism in fish also injected with a low dose of unlabeled T_4.

This looked at the effects of chronic physiological TH challenges on how a low dose of TH in the intestine, which could be encountered in the diet, were handled by the enterohepatic tissues. The T_4 net loss, uptake to the liver or blood, and accumulation in the bile were measured to see if there were alterations in $*T_4$ or $*T_4$ metabolites in EHC tissues. The prediction was that T_4 and T_3 were to a large degree independently regulated. Thus, a dietary T_3 challenge was not expected to alter $*T_4$ metabolism in EHC tissues. This was surmised by comparing amounts of $*T_4$ or $*T_4$ metabolites in each EHC tissue among controls, T_4 TRT, and T_3 TRT.

5. Effects of dietary T_4 or T_3 challenges on $*T_4$ metabolism in fish also injected with a high dose of unlabeled T_4.

The same methods, predictions and tests were used as above, however a high dose of T_4, not a low dose of T_4, accompanied the $*T_4$ injected into the lumen.

6. Effects of dietary T_4 or T_3 challenges on $*T_3$ metabolism in fish also injected with a low dose of unlabeled T_3.

The T_3 net loss, uptake to the liver and the blood, and accumulation in the bile were measured to see if there was an alteration in $*T_3$ or $*T_3$ metabolites in EHC tissues. The prediction was that T_4 and T_3 were to a large degree independently regulated. Thus, a dietary T_4 challenge was not expected to alter $*T_3$ metabolism in EHC tissues. This was surmised by comparing amounts of $*T_3$ or $*T_3$ metabolites in each EHC tissue among controls, T_4 TRT, and T_3 TRT.

7. Effects of dietary T_4 or T_3 challenges on $*T_4$ metabolism in fish also injected with a low dose of unlabeled T_4.

The same methods, predictions, and tests were used as above, however a high dose of T_3, not a low dose of T_3, accompanied the $*T_3$ injected into the lumen.

Statistics

Using the SPSS statistical package, univariate analysis of variance (ANOVA) was used to compare plasma T_4 means or plasma T_3 means for the effects of treatments (C, T_4 TRT, and T_3 TRT) at Day 7. This was followed by LSD post hoc multiple comparisons to determine significant differences among treatments. Independent t-tests were used to compare means for net loss of TH from the intestinal section between T_4 low dose and T_3 low dose or T_4 high dose and T_3 high dose. Independent t-tests were also used to

compare means for net loss of TH from the intestinal section between T_4 low dose and T_4 high dose or T_3 low dose or T_3 high dose. For each component of TH EHC studied (net loss of TH from the intestinal section, uptake by the liver, uptake by the blood, and accumulation in the bile), means were compared for the effects of treatments. Each TH injectate (T_4 low dose, T_4 high dose, T_3 low dose, or T_3 high dose) was analyzed separately. This was followed by LSD post hoc multiple comparisons to determine significant differences among treatments from controls. Results were significantly different at $p \leq 0.05$.

Results

Effects of dietary T_4 and T_3 challenges on plasma TH levels

A dietary T_4 challenge did not significantly change plasma T_4 levels from controls (Figure 6-1). A dietary T_3 challenge increased plasma T_3 (1.58 ng/mL) from controls (0.13 ng/mL), however, the 10-fold increase was not significant ($F=1.026$, d.f.=2, $p=0.362$). This may be largely due to the very high standard error of the T_3-treated fish.

Comparisons of T_4 and T_3 net loss from the intestinal lumen

In control fish, net loss of T_4 low dose from the intestinal section was not significantly different from net loss of T_3 low dose in control fish ($t=1.140$, d.f.=10, $p=0.281$; Tables 6-1 and 6-2). This trend remained the same with dietary TH challenges.

In control fish, net loss of T_4 high dose from the intestinal section was significantly greater than net loss of T_3 high dose ($t=6.949$, d.f.=4, $p=0.002$). This difference in absorption between T_4 and T_3 was abolished with T_3 TRT because a dietary T_3 challenge increased net loss of T_3 high dose.

Effects of a high dose of T_4 or T_3 on T_4 or T_3 net loss from the intestinal lumen

Net loss of $*T_4$ from the intestine did not change significantly with an increase in unlabeled T_4 injected simultaneously (t=0.122, d.f.=6, p=0.907; Table 6-1). Dietary TH challenges of T_4 and T_3 did not affect net loss of T_4 low or high doses (F=0.500, d.f.=2, p=0.617 or F=0.138, d.f.=2, p=0.873, respectively).

Net loss of $*T_3$ from the intestine decreased significantly with an increase in unlabeled T_3 injected simultaneously (t=4.504, d.f.=9, p=0.001; Table 6-2), indicating the presence of a saturable intestinal transport mechanism for T_3 absorption from the intestine. Unlike $*T_4$ net loss, dietary T_4 and T_3 challenges significantly increased $*T_3$ net loss. T_4 TRT increased both net loss of T_3 low dose (F=3.546, d.f.=2, p=0.031) and net loss of T_3 high dose (F=21.325, d.f.=2, p=0.038). Therefore, chronic T_4 did not affect saturability of the T_3 transporter. T_3 TRT did not significantly alter net loss of T_3 low dose (F=3.546, d.f.=2, p=0.349), yet it increased net loss of T_3 high dose (F=21.325, d.f.=2, p=0.000). Therefore, chronic T_3 altered characteristics of T_3 absorption such that net loss of $*T_3$ was not saturable.

Effects of dietary T_4 or T_3 challenges on $*T_4$ metabolism in fish also injected with a low dose of unlabeled T_4

In control fish, 13.2% of the total T_4 low dose injected was lost from the intestinal section (Table 6-1). From the liver, mainly $*T_4$ metabolites entered systemic circulation, while mainly $*T_4$ was excreted to the bile (0.0042%).

With a dietary T_4 challenge, $*T_4$ net loss from the intestine, $*T_4$ uptake to the liver and the blood, and $*T_4$ accumulation in the bile were not altered significantly. However, the amount of $*T_4$ metabolites accumulated in the bile increased significantly to 0.0063%

of the total T_4 injected (F=9.657, d.f.=2, p=0.001), which was comparable to the amount of $*T_4$ accumulated in the bile (0.0062%).

With a dietary T_3 challenge, only $*T_4$ uptake to the blood increased significantly (F=4.148, d.f.=2, p=0.031).

Effects of dietary T_4 or T_3 challenges on $*T_4$ metabolism in fish also injected with a high dose of unlabeled T_4

In control fish, 13.9% of the total T_4 high dose injected was lost from the intestinal section (Table 6-1). A larger portion of $*T_4$ than $*T_4$ metabolites were measured in the liver. From the liver, comparable amounts of $*T_4$ and $*T_4$ metabolites entered the systemic circulation, while mainly $*T_4$ was accumulated in the bile (0.0009%).

With a dietary T_4 challenge, $*T_4$ net loss from the intestine and $*T_4$ uptake to the liver and the blood did not change significantly. Both $*T_4$ (0.0073%) and $*T_4$ metabolite (0.0024%) accumulation in bile increased, but not significantly (F=1.203, d.f.=2, p=0.179 and F=1.209, d.f.=2, p=0.314).

With a dietary T_3 challenge, $*T_4$ net loss from the intestine and $*T_4$ uptake to the liver and the blood, and $*T_4$ accumulation in the bile were unaltered.

Effects of dietary T_4 or T_3 challenges on $*T_3$ metabolism in fish also injected with a low dose of unlabeled T_3

Without dietary TH challenges, 9.7% of total T_3 low dose injected was lost from the intestine (Table 6-2). Comparable amounts of $*T_3$ and $*T_3$ metabolites were measured in the liver. From the liver, less $*T_3$ than $*T_3$ metabolites entered the systemic

circulation. Also from the liver, only *T_3 metabolites were accumulated in the bile (0.0089%).

With a dietary T_4 challenge, *T_3 net loss increased significantly to 16.5% of the total T_3 injected (F=3.546, d.f.=2, p=0.031). *T_3 uptake to liver did not increase significantly. However, *T_3 uptake to blood (F=4.144, d.f.=2, p=0.029) and *T_3 accumulation in bile (0.0025%; F=2.613, d.f.=2, p=0.036) increased significantly.

With a dietary T_3 challenge, *T_3 net loss did not change significantly (9.4%). *T_3 uptake to the liver and blood were not altered significantly. *T_3 accumulated in the bile (0.0017%) increased, but not significantly (F=2.613, d.f.=2, p=0.136). However, *T_3 metabolites accumulated in the bile (0.0009%) decreased significantly (F=2.696, d.f.=2, p=0.030).

Effects of dietary T_4 or T_3 challenges on *T_3 metabolism in fish also injected with a high dose of unlabeled T_3

Without dietary TH challenges, 0.1% of the total *T_3 high dose injected was lost from the intestine (Table 6-2). All of the radioactivity measured in the liver was in the form of *T_3. From the liver, no *T_3 was measured in the blood, only *T_3 metabolites. No radioactivity, *T_3 or *T_3 metabolites, was accumulated in the bile.

With a dietary T_4 challenge, *T_3 net loss from the intestine increased significantly to 8.9% of the total *T_3 injectate (F=21.325, d.f.=2, p=0.038). Both *T_3 and *T_3 metabolites measured in the liver increased, but not significantly. *T_3 uptake to the blood increased significantly (F=4.107, d.f.=2, p=0.015). Radioactivity accumulated in the bile was detected (0.0284%), but the form of radioactivity, *T_3 and/or *T_3 metabolites, was not assayed.

With a dietary T_3 challenge, $*T_3$ net loss from the intestine increased significantly to 22.3% of the total T_3 injectate ($F=21.325$, d.f.=2, $p=0.000$). $*T_3$ measured in the liver was not significantly altered. The amount of $*T_3$ and $*T_3$ metabolites taken up by the blood increased, but not significantly. No $*T_3$ was accumulated in the bile, but $*T_3$ metabolites accumulated in the bile increased significantly to 0.0295% of the total injectate ($F=5.290$, d.f.=2, $p=0.050$).

Discussion

In rainbow trout, the role of the intestine in contributing to the regulation of thyroidal status is unknown. The goal of the present study was to evaluate the potential for TH EHC and the effects of chronic dietary TH challenges on the TH EHC. The Discussion is organized according to the hypotheses presented in the Introduction.

1. Dietary T_4 or T_3 challenges 3-4 days previously did not significantly elevate plasma T_3 nor plasma T_4 levels.

A prior dietary T_3 challenge elevated plasma T_3 levels, though not significantly. A prior dietary T_4 challenge did not elevate plasma T_4 levels. The purpose of feeding TH over a period of 7 days was to create physiological T_4 or T_3 challenges and to determine their effects on the compensatory TH metabolism in the enterohepatic tissues, with the underlying assumption that the effects would persist after 3-4 days of fasting. Based on the results of a similar feeding protocol in Experiment 4, but which was continued until the day before sampling, it was anticipated that there would be no change in plasma T_4 based on a dietary T_4 challenge, but there might be an elevation in plasma T_3 based on a dietary T_3 challenge. In the present experiment, there was indeed no change in plasma T_4.

Although the T_3 challenge that was terminated 3 days prior to sampling did not produce a statistically significant elevation in plasma T_3, it did result in some high plasma T_3 values such that the mean plasma T_3 was over 10-fold that of controls. Therefore, it is clear that even 3 days after the end of feeding, at least some of the T_3-treated fish were still in a period of adjustment to the dietary T_3 challenge. There are no previous studies in trout on the short-term time-course of readjustment in plasma T_3 levels following withdrawal of a chronic dietary T_3 challenge. However, T_3 dependency was found following removal of the dietary T_3 challenge (Eales and Finnson, 1991). Plasma T_3 levels fell to subnormal levels, and enzyme pathways, such as T_4ORD, required at least 6 weeks to return to normal activity levels. Hence, the lack of significant change in plasma levels may represent a rapid regulation of plasma TH levels. Even though trout exhibited this stability of plasma TH levels, other physiological changes, such as enzyme pathways for TH metabolism would still be affected.

A prior dietary TH challenge can alter the physiological state of the trout (Higgs *et al.*, 1979), which influences regulation of TH (Eales *et al.*, 1993), as was addressed in Chapter 4. The main regulation of plasma T_4 is the central control mechanism involving the release of T_4 from the thyroid tissue and its regulation via negative feedback at the hypothalamus and pituitary (Eales *et al.*, 1990; Eales and Himick, 1991). Central control of plasma T_4 levels can occur very quickly, but it will only be as effective as long as the T_4 absorption from the intestine does not exceed the normal rate of T_4 secretion by the thyroid. Beyond this point, the central control cannot compensate for the increase in plasma T_4. Other mechanisms of T_4 regulation may include, but are not limited to, the induced conversion of hepatic T_4IRD which degrades T_4 to rT_3, the deiodination

pathways in the intestinal tissue itself (pathways described in Chapters 3 and 4 which mainly degrade T_4), and increased biliary excretion of T_4 and T_4 metabolites (described in Chapter 5 and a focus of this chapter to be discussed later).

The mechanisms of T_3 autoregulation, which were discussed in Chapter 4, differed from T_4 regulation. Since thyroidal T_3 secretion is not thought to be extensive, the main control of T_3 availability involves peripheral mechanisms of regulation. These include the reduction of hepatic T_4ORD activity which reduces T_3 formation (Eales and Finnson, 1991), possible uptake into muscles, changes in intestinal deiodination (as described in Chapter 4), and increased biliary excretion of T_3 and T_3 metabolites (initially investigated in Chapter 5 and a focus of this chapter to be discussed later).

Thus, the dietary T_4 challenge may have increased compensatory mechanisms and prevented increased plasma T_4 levels. Feeding trout T_3 increased plasma T_3 levels and resulted in a physiological challenge, supporting the hypothesis. After a 3-day cessation of the challenge, many fish compensated, as was detected by some plasma T_3 levels reverting to control levels.

2. Net loss of T_4 from the MI-DI section is greater than net loss of T_3.

When TH net loss was measured, it was assumed that all radioactivity lost from the intestinal section was the intact TH initially injected. This was based on Chapter 5's comparisons of percent TH in the injectate and percent TH remaining in the intestinal section one hour after TH injection into the lumen. Since there was no difference between the two percentages, preferential net loss of $*I^-$ contamination over *TH or intestinal metabolism of *TH did not occur. Thus, TH net loss represented TH absorption from the lumen of the intestinal section.

*T_4 was more readily absorbed from the intestine than *T_3, with a net loss of *T_4 high dose significantly greater than *T_3 high dose. Interestingly, if only plasma TH levels had been measured, then it would have appeared that T_3 was absorbed to a greater extent than T_4. As discussed in Chapter 5, it was previously believed that T_3 was absorbed from the intestine more effectively than T_4. However, measurements of net loss of *TH from the intestinal loop in Chapter 5 and data from this chapter do not support this conclusion. The difference in plasma levels may represent negative feedback of T_4 at the hypothalamo-pituitary level, decreasing thyroidal output of T_4. T_4 exerts a strong negative feedback on the hypothalamo-pituitary-thyroid axis, but T_3 exerts little influence (Eales and Himick, 1991). There may be a difference in plasma TH levels due to clearance pathways that are more sensitive to T_4 than T_3. For example, greater amounts of T_4 are conjugated for excretion to the bile than T_3 (Finnson and Eales, 1997). Together, these regulatory pathways would preferentially reduce plasma T_4 over T_3.

The net loss of *T_3 from the intestine was more sensitive to dietary TH fluctuations than the net loss of *T_4. Dietary TH challenges increased *T_3 net loss to levels of *T_4 net loss. This indicated that the mechanisms of T_4 and T_3 absorption from the intestine differed in sensitivities to the chronic presence of intestinal TH.

Thus, T_4 net loss from the MI-DI section was at least as great as T_3 net loss in all experiments conducted. In fact, T_4 net loss from the intestinal section exceeded T_3 net loss in the majority of the experiments.

3. T_4 and T_3 uptake across the wall of the intestinal lumen do not involve saturable transport systems.

The net loss of $*T_4$ from the intestinal section was not reduced in the presence of a high concentration of T_4, indicating that the mechanism for $*T_4$ net loss was not saturated with 100 pmoles of T_4 injected. $*T_4$ net loss from the intestinal section may be solely by diffusion. However, these data do not exclude a transporter that is saturable at a higher dose of T_4.

In contrast, the net loss of $*T_3$ was reduced in the presence of a 500-fold increase in non-radioactive T_3, indicating that the transport mechanism was saturable at 100 pmoles of T_3 injected. T_3 TRT, but not T_4 TRT, altered the characteristics of T_3 absorption such that net loss of $*T_3$ was no longer saturable. Thus, chronic intestinal exposure to T_3 did not saturate the mechanism of T_3 absorption, rather, it increased the mechanism's ability to transport T_3 from the intestine.

In other tissues, such as hepatocytes and red blood cells, T_4 uptake was described as having non-saturable and saturable components (Riley and Eales, 1994; McLeese and Eales, 1996a, b). Even though characterstics of each tissue's transporters differed, both studies concluded that binding sites for T_4 and T_3 transport differed. The present data also support two separate mechanisms for intestinal T_4 and T_3 net loss. Only $*T_3$ net loss from the intestine responded to chronic influxes of TH, and $*T_4$ net loss from the intestinal lumen was often greater than $*T_3$ net loss. Chronic dietary TH challenges may increase the mass of intestinal tissue, hence its surface area for uptake, and increase the T_3 intestinal transporter itself. However, it is more likely that TH only altered the characteristics of the intestinal transporter, since an increase in intestinal tissue surface

area would inherently alter net loss of *T_4 from the intestine, whether it was based on free diffusion or if it was transport-mediated. TH would be increasing the mucosal layer, and increasing the overall absorptive layer. The TH treatments only increased T_3 net loss from the intestine. Therefore, T_3 net loss from the intestine, not T_4 net loss, was sensitive to prolonged exposure to dietary TH.

Besides having two separate mechanisms for T_4 and T_3 net loss from the intestine, T_3 net loss itself may employ two separate mechanisms. Both dietary T_4 and T_3 challenges increased T_3 net loss. However, a dietary T_4 challenge increased T_3 net loss without altering the transporter's saturability. A dietary T_3 challenge increased T_3 net loss, but the mechanism for absorption was no longer saturable. Chronic T_4 may have up-regulated the T_3 transporter, increasing transport capabilities. Chronic T_3 either increased a non-saturable component of T_3 net loss from the intestine, or the hormone altered the characterstics of the existing transporter, or it induced a transporter that has higher saturability capabilities.

Further information was found for mammals on membrane transport of TH for several tissues, but not for intestinal cells. Two binding sites for each of T_4 and T_3 have been detected in cell membranes and intact cells such as hepatocytes, erythrocytes, kidney, testis, spleen, placenta, and neuroblasts (Hennemann *et al.*, 2001). The mean apparent dissociation constant values (K_d) for high-affinity, low-capacity binding sites for T_4 and T_3 were in the nanomolar range. These transporters bound and translocated TH over membranes. They were often energy- and Na^+-dependent. In contrast, the mean apparent K_d values for low-affinity, high-capacity binding sites for T_4 and T_3 were in the micromolar range. These represented TH binding to membrane proteins, and the process

was energy- and Na^+-independent. Different types of transporters include organic anion transport proteins and amino acid transporters (reviewed in Hennemann *et al.*, 2001; Abe *et al.*, 2002). There are many possible transport mechanisms. The characteristics of transport are determined not only by the type of transport and the efficacy of its translocation but also by variations in the free TH concentrations. Thus, intestinal transport of TH may be determined by various transporters that are sensitive to the fluctuating levels of TH that would be present in the intestinal lumen.

4. A prior dietary T_4 challenge, not a T_3 challenge, will modify aspects of T_4 metabolism by the enterohepatic tissues.

Without dietary TH challenges, the potential for T_4 EHC existed with both a low and a high dose of T_4 injected. $*T_4$ was absorbed from the intestine, and $*T_4$ was accumulated in the bile that would return to the intestine upon refeeding the trout.

A prior dietary T_4 challenge modified aspects of $*T_4$ metabolism by enterohepatic tissues. Chronic T_4 TRT altered T_4 EHC by increasing $*T_4$ metabolites accumulated in the bile after a low dose of T_4 was injected into the intestinal lumen. These changes to T_4 metabolism by enterohepatic tissues were not due to intestinal regulation of T_4 because entry of $*T_4$ into EHC from the intestine was not altered by a chronic influx of T_4 from the diet in the intestine. Presumably the liver regulated the amount of $*T_4$ and $*T_4$ metabolites returning to the intestine through excretion to the biliary pathway, thus altering T_4 EHC. This would decrease the circulating T_4 substrate available for conversion to T_3.

In contrast, a prior dietary T_3 challenge did not modify aspects of $*T_4$ metabolism by enterohepatic tissues. Chronic T_3 TRT did not alter $*T_4$ entry into EHC or $*T_4$ or $*T_4$

metabolites accumulated in the bile. Thus, T_4 regulation was independent from influence by T_3.

In teleost fish, a main control of plasma T_4 levels is through the hypothalamo-pituitary-thyroid axis. T_4 negative feedback occurs at the hypothalamus (Peter, 1971; 1972) and the pituitary (Baker, 1965, 1969a,b; Sage, 1968; Sage and Bromage, 1970). Plasma T_3 does not influence the control of T_4 at any level of the hypothalamo-pituitary-thyroid axis (Eales, *et al.*, 1990; Eales and Himick, 1991). Thus, it is not surprising that the intestine, as a source of T_4, was also insensitive to T_3 control of T_4 net loss.

Overall, a consistent net loss of $*T_4$ from the intestine and supply of T_4 to the intestinal pool occurred. Hence a constant T_4 EHC was maintained, regardless of dietary TH challenges. The ability to maintain a constant T_4 EHC may allow the fish to advantageously utilize a dietary source of TH that would fluctuate greatly. Since T_4 is not the biologically active hormone, an influx of T_4 would not necessarily elicit actions at a target. Peripheral metabolism of T_4 to T_3 and/or T_4 degradation and clearance would be regulated separately.

However, T_4 EHC one hour after injecting T_4 into the intestinal lumen represents an on-going process. A longer residence of the TH within the intestinal lumen may alter the overall results of T_4 EHC. Intestinal metabolism may occur, preventing T_4 net loss from the intestine and entry into T_4 EHC. In studies encompassing longer residence of the injectate in the intestinal lumen, T_4 was metabolized to iodide, and T_4 itself did not enter systemic circulation (Whitaker and Eales, 1993). In Chapter 4, dietary TH challenges increased intestinal metabolism. The intestinal T_4 metabolism pathways (T_4cORD, T_4ORD, T_4IRD, and T_4IP) were characterized as mainly degradative,

supporting findings in other studies that T_4 may be poorly absorbed from the intestine. Thus, comparisons to other experiments should consider the context of time and methodology. Again, for this experiment, T_4 EHC existed within one hour after injection of T_4 into the intestinal lumen.

5. A prior dietary T_3 challenge, not a dietary T_4 challenge, will modify aspects of T_3 metabolism by the enterohepatic tissues.

Without dietary TH challenges, there was no T_3 EHC with both a low and a high dose of T_3 injected. $*T_3$ was absorbed from the intestine, but no $*T_3$, only $*T_3$ metabolites, were accumulated in the bile. Both dietary T_3 and T_4 challenges modified aspects of T_3 metabolism by the enterohepatic tissues.

A prior dietary T_3 challenge modified aspects of $*T_3$ metabolism by enterohepatic tissues. Chronic T_3 TRT increased $*T_3$ entry into EHC by increasing $*T_3$ net loss from the intestinal section. Chronic T_3 TRT increased $*T_3$ metabolites accumulated in the bile after a low dose of T_3 injected into the intestinal lumen. However, this did not create T_3 EHC with at a high T_3 dose, since no intact T_3 returned to the intestine. Thus, a prior dietary T_3 challenge created T_3 EHC at the low dose only. Otherwise, the intestine increased disposal of $*T_3$ metabolites.

Regulation of T_3 EHC may be more sensitive to acute changes in T_3 levels for several reasons. First, T_3 supply in trout occurs through tissue demand and not through thyroidal supply (Eales and Brown, 1993). T_3 is the biologically active hormone, and its availability is regulated by T_4 to T_3 conversion at peripheral tissues. Thus, a continual supply of T_3 through T_3 EHC would represent a source of T_3 not driven by tissue demand. Second, this would be a situation encountered in the wild that the trout would be adapted

to: variable influxes of TH when trout eat other fish, including their TH. Since T_3 is the biologically active hormone, its availability to the whole system would be an immediate concern that would not allow time for enzyme synthesis necessary for TH metabolism. Hence, the present study indicated that T_3 transport was saturable. Increased T_3 was not free to diffuse in and increase circulating levels. After injecting a high dose of T_3, the T_3 that entered was not retained in blood to distribute to the whole body, and T_3 was not accumulated in the bile. These suggest that other excretory routes (branchial or urinary systems) may be stimulated to increase plasma clearance rates, handling influxes of $*T_3$ and $*T_3$ metabolites.

For trout, up to 15% of injected $*T_4$ and 12.3% of injected $*T_3$ can be excreted into urine, while up to 41.4% of $*T_4$ and 25.7% of $*T_3$ can be excreted into the gall bladder (Parry *et al.*, 1994). Thus, urine is a significant route for excretion of TH and TH conjugates. Yet, renal excretion was not regulated to compensate for TH challenge. In addition, up to 25% of TH was not accounted for by excretion into urine or gall bladder.

Other excretory routes or storage pools include gills and muscle. Rapid uptake of TH by gills from water (Eales, 1974; Omeljaniuk and Eales, 1985) also suggested that exchange across the gills can occur. Skeletal muscle, a possible storage site, comprised the largest tissue pool for T_3, and it was also responsive to altered physiological states such as low pH and increased aluminum exposure (Fok *et al.*, 1990). These excretory routes and storage sites together may regulate an acute T_3 high dose.

Unexpectedly, a prior dietary T_4 challenge also modified aspects of $*T_3$ metabolism by enterohepatic tissues. Chronic T_4 TRT increased T_3 entry into EHC by increasing $*T_3$ absorption from the intestine. Chronic T_4 TRT also altered the potential

T_3 EHC by increasing *T_3 accumulated in the bile after a low dose of T_3 was injected into the intestinal lumen. Overall, T_4 TRT stimulated T_3 EHC, and the changes to T_3 EHC included both intestinal and hepatic regulation of T_3 metabolism in response to chronic influxes of intestinal T_4.

The increase in T_3 accumulated in the bile may be due to the effects of increased T_4 on the liver. Finnson and Eales (1997) have shown that T_4 can inhibit T_3 glucuronidation. In the present experiment, *T_3 metabolites accumulated decreased slightly with T_4 treatment, yet it was enough to significantly increase *T_3 accumulation. Any excess *T_3 not accumulated in the bile would appear in the blood, hence the increased *T_3 blood uptake was measured.

Overview

Overall, chronic dietary TH challenges, both T_4 and T_3, increased T_3 EHC. However, dietary TH challenges increase intestinal metabolism (Chapter 4). Specifically, a dietary T_3 challenge increased T_4cORD, T_4IRD, T_4IP, and T_3ORD in the MI, decreased T_4ORD in the MI, and increased T_4ORD and T_3IRD in the DI. All of these pathways together were degradative pathways, preventing the formation of T_3. Thus, increased return of T_3 to the intestine would not necessarily supply the intestinal pool for T_3 EHC. Rather, it would be an increased T_3 excretory route through the intestine. Since T_4 treatment provided excess T_4 substrate for conversion to T_3, and T_3 treatment provided biologically active T_3, an increase in T_3 return to the intestine for excretion would regulate thyroidal status by preventing excess T_3.

In the previous chapter, high TH doses, both T_4 and T_3, resulted in only TH accumulated in the bile. Yet, this pattern of TH return to the intestine was not seen here,

especially for T_3. In these experiments, the fish are larger, reproductively mature, and they tend to have lower circulating levels of TH, lower metabolism, and lower demands for TH. This situation may create a lower need for TH EHC, especially T_3, to regulate thyroidal status in larger trout.

Conclusions

From examination of the above hypotheses, several conclusions may be drawn. Prior dietary TH challenges may have increased compensatory mechanisms. After a 3-day cessation of the dietary T_4 challenge, plasma T_4 levels were not elevated. A prior dietary T_3 challenge elevated plasma T_3 levels, but it was not statically significant. After a 3-day cessation of the prior dietary T_3 challenge, many fish compensated, as was detected by some plasma T_3 levels reverting to control levels.

T_4 net loss from the intestinal section was greater than that for T_3. However, dietary TH challenges increased T_3 net loss from the intestine to amounts of net loss measured for T_4. There was no evidence for a saturable T_4 transporter for T_4 net loss from the intestine. This did not change with dietary TH challenges. However, there was evidence for a saturable T_3 transporter for T_3 net loss from the intestine. Dietary T_4 challenges and dietary T_3 challenges, to a greater extent, increased T_3 net loss and changed transport characterstics so that T_3 net loss was no longer saturable. Both differential T_4 and T_3 net loss from the intestine and differential T_4 and T_3 net loss resulting from dietary TH challenges suggested separate transport mechanisms (if a transporter is involved for T_4) for T_4 and T_3 in the intestine.

With TH absorption from the intestine and accumulation in the bile, the potential for TH EHC and the effects of chronic dietary TH challenges on the potential TH EHC

were evaluated. For controls, T_4 EHC existed. A prior dietary T_4 challenge, but not a T_3 challenge, modified aspects of T_4 metabolism. The liver regulated the increase in T_4 metabolites returning to the intestine for T_4 low dose injected. Thus, excess T_4 was metabolized, removing the substrate for conversion to biologically active T_3. This would work in conjuction with negative feedback to prevent fluctuations in plasma T_4 levels. For controls, T_3 EHC did not exist. A prior dietary T_3 challenge modified aspects of T_3 metabolism through intestinal and hepatic regulation. However, T_3 EHC existed only for a low dose of T_3 injected into the intestine after dietary T_4 or T_3 challenges. The intestine and the liver increased the T_3 EHC.

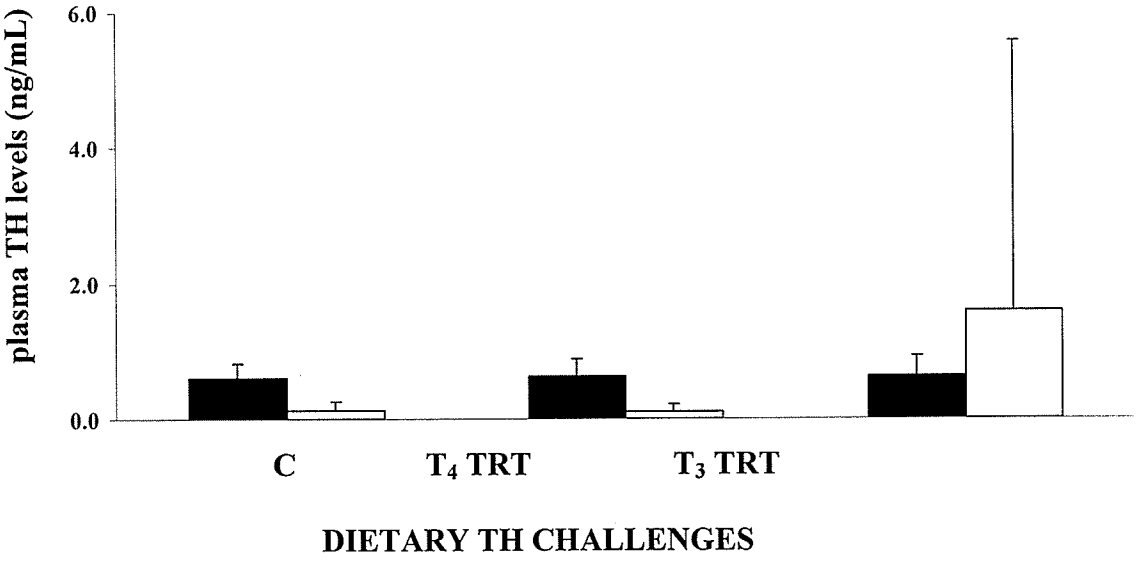


Figure 6-1. Plasma T_4 levels (■) and plasma T_3 levels (□) after 7 days of dietary TH challenges for control fish (C, n=32), fish fed 12 ppm T_4 (T_4 TRT, n=33), and fish fed 12 ppm T_3 (T_3 TRT, n=36). Bars represent means (± s.e.).

Table 6-1. Effects of 7 days of dietary T_4 challenge (T_4 TRT) or dietary T_3 challenge (T_3 TRT) on the fate of *T_4 injected one hour previously into the lumen of the intestinal section, accompanied by either a low dose (200 fmoles) or a high dose (100 pmoles) of unlabeled T_4. Numbers represent percent total injectate/mg of tissue x g of body weight/500 g (%/mg) ± s.e. Letters indicate significant differences at $p \leq 0.050$.

		T_4 LOW	T_4 HIGH
NET LOSS (%)*	**Control**	13.2 ± 3.7 (n=9)	13.9 ± 5.3 (n=6)
	T_4 TRT	11.8 ± 1.0 (n=9)	11.2 ± 1.3 (n=5)
	T_3 TRT	10.1 ± 2.0 (n=9)	14.6 ± 7.7 (n=6)
UPTAKE TO LIVER (%/mg)			
TH	**Control**	3.1 ± 0.8	8.9 ± 5.4
	T_4 TRT	2.2 ± 0.7	15.2 ± 10.3
	T_3 TRT	4.0 ± 1.0	12.3 ± 5.6
TH METABOLITES	**Control**	4.5 ± 1.1	1.9 ± 1.2
	T_4 TRT	6.5 ± 2.1	7.8 ± 5.3
	T_3 TRT	2.3 ± 0.6	5.4 ± 2.5
UPTAKE TO BLOOD (%/mg)			
TH	**Control**	1.1 ± 0.2	1.1 ± 1.0
	T_4 TRT	0.6 ± 0.1	4.0 ± 2.1
	T_3 TRT	4.7 ± 1.9[b]	4.6 ± 2.7
TH METABOLITES	**Control**	10.5 ± 2.2	1.7 ± 1.5
	T_4 TRT	11.4 ± 2.7	13.2 ± 7.0
	T_3 TRT	9.3 ± 3.7	9.0 ± 5.2
ACCUMULATION IN BILE (%)*			
TH	**Control**	0.0042 ± 0.0016	0.0009 ± 0.0009
	T_4 TRT	0.0062 ± 0.0017	0.0073 ± 0.0062
	T_3 TRT	0.0029 ± 0.0018	0.0016 ± 0.0010
TH METABOLITES	**Control**	0.0004 ± 0.0001	0.0001 ± 0.0001
	T_4 TRT	0.0063 ± 0.0017[a]	0.0024 ± 0.0020
	T_3 TRT	0.0003 ± 0.0002	0.0008 ± 0.0004

[a] $p=0.001$ C vs. T_4 TRT

[b] $p=0.031$ C vs. T_3 TRT

* units in percent of total injectate (%), instead of %/mg

Table 6-2. Effects of 7 days of dietary T_4 challenge (T_4 TRT) or dietary T_3 challenge (T_3 TRT) on the fate of *T_3 injected one hour previously into the lumen of the intestinal section, accompanied by either a low dose (200 fmoles) or high dose (100 pmoles) of unlabeled T_3. Numbers represent percent total injectate/mg of tissue x g of body weight/500 g (%/mg) ± s.e. N.A. denotes not assayed. Letters indicate significant differences at $p \leq 0.050$.

		T_3 LOW	T_3 HIGH
NET LOSS (%)*	**Control**	9.7 ± 1.3 (n=9)	0.1 ± 0.1[a] (n=3)
	T_4 TRT	16.5 ± 2.1[b] (n=8)	8.9 ± 1.4[c] (n=5)
	T_3 TRT	9.4 ± 3.3 (n=9)	22.3 ± 2.9[d] (n=6)
UPTAKE TO LIVER (%/mg)			
TH	**Control**	9.6 ± 4.3	7.5 ± 7.5
	T_4 TRT	7.9 ± 2.5	21.7 ± 11.1
	T_3 TRT	2.5 ± 0.9	3.2 ± 0.9
TH METABOLITES	**Control**	11.2 ± 5.0	0
	T_4 TRT	5.1 ± 1.6	3.5 ± 1.8
	T_3 TRT	1.7 ± 0.6	1.2 ± 0.3
UPTAKE TO BLOOD (%/mg)			
TH	**Control**	2.4 ± 1.0	0
	T_4 TRT	7.0 ± 2.2[e]	13.4 ± 3.8[h]
	T_3 TRT	1.8 ± 0.7	8.0 ± 2.3
TH METABOLITES	**Control**	12.1 ± 4.8	6.3 ± 6.3
	T_4 TRT	8.9 ± 2.8	20.0 ± 5.7
	T_3 TRT	2.9 ± 1.1	5.1 ± 1.5
ACCUMULATION IN BILE (%)*			
TH	**Control**	0	0
	T_4 TRT	0.0025 ± 0.0010[f]	0.0284 ± 0.0271†
	T_3 TRT	0.0017 ± 0.0010	0
TH METABOLITES	**Control**	0.0089 ± 0.0039	0
	T_4 TRT	0.0042 ± 0.0016	N.A.
	T_3 TRT	0.0009 ± 0.0005[g]	0.0295 ± 0.0088[i]

[a] $p=0.001$ T_3 low vs. high
[b] $p=0.031$ C vs. T_4 TRT
[c] $p=0.038$ C vs. T_4 TRT
[d] $p=0.000$ C vs. T_3 TRT
[e] $p=0.029$ C vs. T_4 TRT
[f] $p=0.036$ C vs. T_4 TRT
[g] $p=0.030$ C vs. T_3 TRT
[h] $p=0.015$ C vs. T_3 TRT
[i] $p=0.050$ C vs. T_3 TRT

† total radioactivity accumulated since *TH and *TH metabolites were not assayed

* units in percent of total injectate (%), instead of %/mg.

Chapter 7

General Discussion

This study was performed to determine the properties and capacities of the various regions of the rainbow trout intestine to metabolize TH, absorb TH from the lumen, complete the TH EHC through TH uptake to the liver, systemic blood, and gall bladder, and regulate the above pathways following physiological challenges with dietary T_4 and T_3.

Intestinal TH metabolism pathways were more complex than those in the liver, and the intestine showed a broader array of TH deiodination and metabolism pathways geared toward degradation of T_4 and T_3. The active intestinal TH metabolism pathways were T_4cORD which completely deiodinated T_4 on the outer ring, T_4ORD which monodeiodinated T_4 on the outer ring to T_3, T_4IRD which monodeiodinated T_4 on the inner ring to rT_3, T_3ORD which monodeiodinated T_3 on the outer ring to 3,5-T_2, T_3IRD which monodeiodinated T_3 on the inner ring to 3,3'-T_2, and IP production which formed iodinated proteins from T_4 or T_3. In the PC, few TH metabolism pathways were active, except for T_4cORD. In the MI, mainly T_4 metabolism pathways were active. The DI was the most active region of the intestine. The predominant pathways were T_4cORD and T_3ORD, however, all of the TH metabolism pathways were active. Together, these pathways would deiodinate T_4 and T_3, removing a potential source of biologically active T_3 and salvaging I^- before loss through excretion.

Besides exhibiting a broader array of active TH metabolism pathways than the liver, the assay cofactor requirements for the intestine also differed from those of the liver. The

intestinal TH metabolism pathways were active without DTT, and in fact, T_4cORD, T_3ORD, and TH IP were more active in its absence. Since the cofactor requirements for the intestine were different than for the liver, and since different TH metabolism pathways were active, the intestine may have evolved different enzyme characteristics along with different roles in regulating TH metabolism.

The difference between the liver and the intestine under physiological challenges of T_4 and T_3 indicated their different roles in the regulation of TH. Under normal, non-stressful conditions, the liver is a main peripheral regulator of thyroidal status. The predominant pathway for maintaining available T_3 is T_4ORD (Eales, *et al.*, 1993), as was seen in this study. To maintain T_3 homeostasis in response to a dietary T_3 challenge, the liver decreased T_4ORD and increased T_4IRD. The pathways reduced production of T_3 and removed the substrate T_4.

In contrast, the intestine, specifically the MI and DI, moderated influxes of TH from the intestinal lumen. A dietary T_4 challenge did not result in increased plasma T_4 levels. This can be accounted for by both central regulation and also by peripheral changes in TH metabolism by the intestine. The DI responded by increasing T_4ORD. However, T_4cORD and T_4IRD were consistently active with or without physiological challenges. Thus, the plasma pool of T_4 available to tissues was strictly maintained by negative feedback controlling thyroidal output and by TH metabolism controlling intestinal uptake.

A dietary T_3 challenge resulted in increased plasma T_3 levels. T_3 would not be regulated centrally but rather by peripheral mechanisms. Both MI and DI regions responded to increased dietary T_3 by degrading T_3 or preventing the formation of new T_3. In the MI, T_4cORD, T_4IRD, T_4IP, and T_3ORD increased. In the DI, T_4ORD and T_3IRD

increased. Thus, the MI and DI regions of the intestine that would have access to both increased TH from the diet and biliary discharge were sensitive to the presence of chronic T_3, and the regions increased their metabolism of TH. The intestine contributed to the autoregulation of T_3 by preventing potential surges of TH to the systemic circulation. Since intestinal TH metabolism was greatest in the DI, this region may salvage I^- before loss through excretion.

In the MI section, the greatest net loss from the intestine was measured for T_3. However, the MI section did not contribute to T_4 EHC or T_3 EHC. With an increase in T_4 or T_3 injected into the intestinal lumen, both TH were absorbed, however, peripheral tissues quickly metabolized and cleared TH via the biliary route, maintaining low levels of T_4 and T_3 in systemic circulation. The MI and other peripheral tissues together prevent fluctuations of TH in the circulation, especially T_3, that may enter from the MI from both dietary and biliary sources.

In the MI-DI section, the greatest net loss from the intestine was measured for T_4. In addition, T_4 EHC was greater than T_3 EHC, due to more unconjugated T_4 than T_3 returning to the intestine in the bile. An acute increase in T_4 and T_3 injected increased the proportion of TH accumulated in the bile to 100% TH, increasing both T_4 and T_3 EHC. Thus, the MI and DI regions of the intestine together may cycle TH either to provide an extrathyroidal source of TH or to allow the intestine to salvage I^- before loss through excretion.

The MI-DI section was further investigated. The net loss of T_4 from the intestine was greater than net loss of T_3, and it was not saturable at 100 pmoles of T_4 injected. In addition, dietary challenges of T_4 and T_3 did not alter the net loss of T_4. Thus, potentially

large, unregulated amounts of T_4 can enter into circulation from the intestine. Previous speculations on the absorption of TH from the intestine focused on the greater entry of T_3 into systemic circulation than T_4. However, the present study supports greater net loss of T_4 from the intestine than T_3, at least within one hour. The previous conclusions on poor T_4 uptake from the intestine may be invalid, since the method of measuring absorption was based on measuring plasma TH levels. As discussed earlier, central control mechanisms are sensitive to plasma T_4 levels, and they compensate for increases in circulating T_4, regardless of its source.

Unlike T_4 net loss from the intestine, T_3 net loss was saturable at 100 pmoles of T_3 injected. This indicates that there are separate mechanisms for T_4 and T_3 absorption, and that the amount of T_3 entering the intestine into circulation is regulated. With a dietary T_3 challenge, T_3 net loss appears to be non-saturable at a high T_3 dose injected. However, the transport mechanism for absorption may have been saturated with the chronic T_3 challenge. Since intestinal TH metabolism pathways are activated with a dietary T_3 challenge, and only net loss of total radioactivity can be measured, increased absorption may be $^*I^-$ released from TH metabolism, not the $^*T_3$ injected.

Dietary TH challenges, even 3 days previously, modified aspects of TH metabolism and altered TH EHC. A prior dietary T_4 challenge increased the proportion of T_4 in the bile returning to the intestine as T_4 metabolites. Thus, the T_4 EHC was altered through peripheral metabolism to decrease the circulating T_4 substrate available for conversion to T_3. A prior dietary T_4 challenge also altered T_3 EHC but by increasing the amount of TH in the bile returning to the intestine. Without TH treatments, T_3 EHC was non-existent. The effects of T_4 on T_3 regulation may actually be from an increase in T_4 substrate

causing an increase in T_3 production. Thus, some effects on T_3 EHC would be expected as T_3 increases. This was confirmed with a prior dietary T_3 challenge which also increased T_3 EHC by increasing TH in the bile returning to the intestine.

Intestinal TH metabolism, enteric absorption of TH, and TH EHC may represent a large store of endogenous and exogenous TH available to the fish, or they may represent salvaging of I^- from this large store of TH. Thyroid function depends on I^-, which could be limiting in the freshwater environment of the rainbow trout. Thus, the DI may have evolved as the region of the intestine to recycle TH and salvage I^- before loss through excretion. This would contribute to other adaptations of the rainbow trout in obtaining and retaining I^- at high levels in the plasma (Gregory and Eales, 1975; Eales and Brown, 1993).

Overall, the intestine is more sensitive to increases in T_3, and it has mechanisms to prevent large increases in systemic T_3: TH metabolism pathways increase; T_3 absorption is saturable; and T_3 EHC increases. It appears that central control is a main control for T_4 availability. However, intestinal TH metabolism and other peripheral metabolism aid in reducing the amount of T_4 absorbed from the intestine and also returning to the intestine. This differential regulation of T_4 and T_3 may reflect a primitive control of T_3 that was retained in teleost fish. Peripheral control, with the intestine playing a major role in T_3 availability, evolved early on in primitive fish (Eales, 1997). In lampreys, no central control of thyroid hormones has been discovered, and peripheral control may be the main regulator of thyroidal status (Dickoff and Darling, 1983). In fact, intestinal TH metabolism is greater than hepatic TH metabolism in more primitive fish, possibly due to this emphasis on peripheral control of T_3 by the intestine (Eales *et al.*, 1997, 1999;

McLeese *et al.*, 2000). A more recently evolved central control of T_4 is seen in more advanced fish, and this trend of further evolving central control can be followed through the vertebrate classes to mammals. Thus in rainbow trout, the intestinal control of TH metabolism and the regulation of thyroidal status represents both a primitive intestinal control of T_3 and T_4 metabolism and absorption with a more derived central control of T_4.

To further elucidate the role of the intestine in TH metabolism and the regulation of thyroidal status, future studies should focus on the differential regulation of T_4 and T_3, especially the entry of TH into circulation. This could be studied by using enterocytes to determine mechanisms for T_4 and T_3 transport and the conditions altering this transport.

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Appendix I

Used to calculate total *T_4 metabolism in pmoles T_4 converted per hour per mg protein.

Numbers from assay:

cpm of *T_4 metabolism product (MET) = _____

cpm of *T_4 substrate in assay (TCR) = _____

contamination of *T_4 (CONT) = _____

specific activity of *T_4 (S.A.) = _____

decay factor of *T_4 (D.F.) = _____

unlabeled substrate (K value) = _____
(where 0.02 nM = 0.3K, 0.16 nM = 2.5K, 1.29 nM = 20K)

Numbers predetermined:

2 = correction for unlabeled I^- removed not detected in radioactive counts

5.2 = correction for dilution in assay volume

1,540,000 = cpm for 1 μCi (2,200,000) corrected for efficiency of gamma counter (70%)

777 = molecular weight of T_4

Calculations:

corrected TCR (cTCR)	= TCR - (CONT x 5.2)
corrected S.A. (cS.A.)	= S.A. x D.F.
radioactive substrate (rSUB)	= 1000/(cS.A. x cTCR)/1,540,000
total substrate (tSUB)	= (rSUB + (K value/10)) x (1000/777)
constant	= ((tSUB x 2 x 5.2)/cTCR) x (60/min of incubation)
Total *T_4 metabolism	**= MET x constant/mg protein**

Appendix II

Used to calculate total *T_3 metabolism in pmoles T_3 converted per hour per mg protein.

Numbers from assay:

cpm of *T_3 metabolism product (MET) = _____

cpm of *T_3 substrate in assay (TCR) = _____

contamination of *T_3 (CONT) = _____

specific activity of *T_3 (S.A.) = _____

decay factor of *T_3 (D.F.) = _____

unlabeled substrate (K value) = _____
(where 0.02 nM = 0.3K, 0.19 nM = 2.5K, 1.52 nM = 20K)

Numbers predetermined:

5.2 = correction for dilution in assay volume

1,540,000 = cpm for 1 μCi (2,200,000) corrected for efficiency of gamma counter (70%)

652 = molecular weight of T_3

Calculations:

corrected TCR (cTCR)	= TCR - (CONT x 5.2)
corrected S.A. (cS.A.)	= S.A. x D.F.
radioactive substrate (rSUB)	= 1000/(cS.A. x cTCR)/1,540,000
total substrate (tSUB)	= (rSUB + (K value/10)) x (1000/652)
constant	= ((tSUB x 5.2)/cTCR) x (60/minutes of incubation)
Total *T_3 metabolism	**= MET x constant/mg protein**

Appendix III

Used to calculate IP production in pmoles TH converted to IP per hour per mg protein.

Numbers from assay:

cpm of *TH IP product (MET) = _____

cpm of *TH substrate in assay (TCR) = _____

contamination of *TH (CONT) = _____

specific activity of *TH (S.A.) = _____

decay factor of *TH (D.F.) = _____

unlabeled substrate (K value) = _____
(where 0.02 nM = 0.3K, 0.16 nM = 2.5K, 1.29 nM = 20K)

Numbers predetermined:

5.2 = correction for dilution in assay volume

1,540,000 = cpm for 1 μCi (2,200,000) corrected for efficiency of gamma counter (70%)

777 = molecular weight of T_4

652 = molecular weight of T_3

Calculations:

corrected TCR (cTCR)	= TCR - (CONT x 5.2)
corrected S.A. (cS.A.)	= S.A. x D.F.
radioactive substrate (rSUB)	= 1000/(cS.A. x cTCR)/1,540,000
total substrate (tSUB)	= (rSUB + (K value/10)) x (1000/mole. wt. of TH)
constant	= ((tSUB x 2 x 5.2)/cTCR) x (60/min of incubation)
TH IP production	**= MET x constant/mg protein**

Appendix IV

Used to calculate activities of specific *T_4 metabolism pathways in pmoles T_4 converted to product per hour per mg protein.

Numbers from assay:

area under the curve for specific *T_4 metabolism product (MET) = _____

cpm of *T_4 substrate in assay (TCR) = _____

contamination of *T_4 (CONT) = _____

specific activity of *T_4 (S.A.) = _____

decay factor of *T_4 (D.F.) = _____

unlabeled substrate (K value) = _____
(where 0.02 nM = 0.3K, 0.16 nM = 2.5K, 1.29 nM = 20K)

Numbers predetermined:

2 = correction for unlabeled I^- removed not detected in radioactive counts

1,540,000 = cpm for 1 μCi (2,200,000) corrected for efficiency of gamma counter (70%)

777 = molecular weight of T_4

Calculations:

corrected TCR (cTCR)	= TCR - (1 - CONT)
corrected S.A. (cS.A.)	= S.A. x D.F. (1 - CONT)
radioactive substrate (rSUB)	= 1000/(cS.A. x cTCR)/1,540,000
total substrate (tSUB)	= (rSUB + (K value/10)) x (1000/777)
constant	= (tSUB)/cTCR) x (60/minutes of incubation)

Specific *T_4 metabolism pathway = MET x constant/mg protein

Appendix V

Used to calculate activities of specific T_3 metabolism pathways in pmoles T_3 converted to product per hour per mg protein.

Numbers from assay:

area under the curve for specific *T_3 metabolism product (MET) = _____

cpm of *T_3 substrate in assay (TCR) = _____

contamination of *T_3 (CONT) = _____

specific activity of *T_3 (S.A.) = _____

decay factor of *T_3 (D.F.) = _____

unlabeled substrate (K value) = _____
(where 0.02 nM = 0.3K, 0.19 nM = 2.5K, 1.52 nM = 20K)

Numbers predetermined:

1,540,000 = cpm for 1 μCi (2,200,000) corrected for efficiency of gamma counter (70%)

652 = molecular weight of T_3

Calculations:

corrected TCR (cTCR)	= TCR - (1 - CONT)
corrected S.A. (cS.A.)	= S.A. x D.F. x (1 - CONT)
radioactive substrate (rSUB)	= 1000/(cS.A. x cTCR)/1,540,000
total substrate (tSUB)	= (rSUB + (K value/10)) x (1000/652)
constant	= (tSUB) x (60/minutes of incubation)

Specific *T_3 metabolism pathways = MET x constant/mg protein

Appendix VI

Used to calculate T_4cORD activity in pmoles T_4 converted to product per hour per mg protein.

Numbers from assay:

area under the $*I^-$ curve (MET) = _____

cpm of $*T_4$ substrate in assay (TCR) = _____

contamination of $*T_4$ (CONT) = _____

specific activity of $*T_4$ (S.A.) = _____

decay factor of $*T_4$ (D.F.) = _____

unlabeled substrate (K value) = _____
(where 0.02 nM = 0.3K, 0.16 nM = 2.5K, 1.29 nM = 20K)

Numbers predetermined:

2 = correction for unlabeled I^- removed not detected in radioactive counts

1,540,000 = cpm for 1 μCi (2,000,000) corrected for efficiency of gamma counter (77%)

777 = molecular weight of T_4

Calculations:

corrected TCR (cTCR)	= TCR - (1 - CONT)
corrected S.A. (cS.A.)	= S.A. x D.F. x (1 - CONT)
radioactive substrate (rSUB)	= 1000/(cS.A. x cTCR)/1,540,000
total substrate (tSUB)	= (rSUB + (K value/10)) x (1000/777)
constant	= (tSUB x 2) x (60/minutes of incubation)
Total I^- production	= MET x constant/mg protein
T_4ORD activity (from Appendix V)	
T_4cORD activity	**= Total I^- production – T_4ORD activity**

Appendix VII

Correction factors for blood, plasma, liver, bile, and intestinal loop tissues which were calculated from adding a known amount of $*T_4$ to each tissue, extracting radioactivity, and eluting *TH from LH-20 columns. The injectate was also eluted from LH-20 columns to serve as a standard reference.

TISSUE	$*T_4$ RECOVERY (%)	CORRECTION FACTOR
BLOOD	58.4	1.7123
PLASMA	67.9	1.4728
LIVER	69.8	1.4327
BILE	30.5	3.2787
LOOP	80.5	1.2430
INJECTATE	100	1.0000

Appendix VIII

Correction factors for blood, plasma, liver, bile, and intestinal loop tissues which were calculated from adding a known amount of $*T_3$ to each tissue, extracting radioactivity, and eluting *TH from LH-20 columns. The injectate was also eluted from LH-20 columns to serve as a standard reference.

TISSUE	$*T_3$ RECOVERY (%)	CORRECTION FACTOR
BLOOD	36.5	2.7397
PLASMA	58.8	1.7007
LIVER	94.3	1.0604
BILE	27.2	3.6765
LOOP	85.6	1.1682
INJECTATE	100	1.0000

Appendix IX

Equations used for linear regression analyses (unbalance ANOVA) to compare TH metabolism activities for the effects of treatments (i; C, T_4 TRT, and T_3 TRT) and time (j; Day 0, 2, and 7) and Tukey's Multiple Comparison used to compute confidence intervals and determine significant differences among treatments over time.

i = TRT:	x1	x2	j = DAY:	x3	x4
C	1	0	Day 0	1	0
T_3 TRT	-1	1	Day 2	-1	1
T_4 TRT	0	-1	Day 7	0	-1

i = TRT
j = DAY
m = response in a cell
a = 3
b = 3

$$\mathbf{Y_{ijm} = \mu\ldots\alpha_1x_1+\alpha_2x_2+\beta_1x_3+\beta_2x_4+(\alpha\beta)_{11}x_1x_3+(\alpha\beta)_{12}x_1x_4+(\alpha\beta)_{21}x_2x_3+(\alpha\beta)_{22}x_2x_4+\Sigma_{ijm}}$$

Testing hypothesis with reduced models:

$$F^* = \frac{SSE(\text{reduced model}) - SSE(\text{full model})}{df_{error}(\text{reduced model}) - df_{error}(\text{full model})}$$

if $F^* \leq F(.984; (df_{error}(\text{reduced}) - df_{error}(\text{full}), df_{error}(\text{full}))$
then conclude H_0: no significant effect.

Look at pairwise comparisons of factor levels means: Tukey's Multiple Comparison

Estimator example: $\mu_i = \sum_{i}^{b} Y_i/b$

Confidence intervals:

$(\mu_1 - \mu_2) \pm 1/(\text{sq root } 2) \times q(1 - \alpha; a, n_T - ab) \times \text{sq root } (MSE/b^2 \sum_{j}^{b} 1/n_{1j} + 1/n_{2j})$

$(\mu_1 - \mu_3) \pm$ same

$(\mu_2 - \mu_3) \pm$ same