PROPERTIES OF 5'-MONODEIODINASE AND FACTORS THAT AFFECT ITS ACTIVITY IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

BY

DEBORAH L. MACLATCHY

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

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FOR

WILLIAM STEVENSON CAMPBELL

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ABSTRACT

The enzyme, 5'-monodeiodinase (5'D), which converts the thyroid pro-hormone thyroxine (T_4) to the active hormone 3,5,3'-triiodothyronine in rainbow trout (Oncorhynchus mykiss), was partially characterized, and the effects on its activity by testosterone, estradiol, growth hormone and diet were investigated. 5'D activity was determined in the microsomal fraction of various tissue(s) by the loss of $^{125}I^-$ from [^{125}I]T_4.

Activity of 5'D was established at a low T_4 range in liver, gill and muscle, and at a high T_4 range in liver and kidney. In the low substrate range (0.08 - 1.3 nM), K_m values were: liver, 0.1 nM, gill 0.2 nM, muscle, 0.17 nM. dithiothreitol (DTT) increased both reaction velocity and the K_m, and propylthiouracil (PTU) uncompetitively inhibited the reaction, suggesting a ping-pong mechanism. In the high substrate range (1.6 - 25 nM), liver and kidney K_m values were 10 and 15 nM respectively. DTT increased the velocity and PTU had no effect, indicating a sequential mechanism. These results indicate that there are two 5'D systems located in various trout tissues.

Testosterone, methyl testosterone and testosterone propionate were injected intraperitoneally (ip) into arctic charr (Salvelinus alpinus), and 17β-estradiol (E2) was injected into trout (0.5 mg/100μg), on days 0 and 3, and

sampled on days 7 and 12. Plasma androgen, T_3 and V_{max} levels were elevated by all androgen treatments; K_m changes were negligible. Following E2 treatment, V_{max} was depressed on Day 7, but had recovered to control levels by Day 12. Injection (ip) of trout with 0.04 μg/g human growth hormone (hGH) increased plasma levels of T_3 and V_{max} of 5'D as early as 8 h post-injection (pi), in a dose-dependent manner. Maximal stimulation occurred at 24 h pi. No effects on K_m or plasma T_4 were measured. Salmon GH injected ip (0.1 or 0.5 μg/g) increased plasma T_3 and 5'D V_{max} at 24 h pi. Previously documented effects of these hormones on the thyroid system can in part now be explained by their effects on 5'D.

Trout were fed isocaloric and isonitrogenous diets with varying lipid/carbohydrate ratios, supplemented with 0, 4, 8, or 12 ppm T_3. V_{max} was unaffected by lipid/carbohydrate ratio, but T_3 progressively decreased the V_{max} of 5'D. Trout were also fed isocaloric diets with either varying protein/lipid ratios or with varying protein/carbohydrate ratios. V_{max} levels were significantly greater with greater protein. Values of K_m were unaffected by any of the diets. Protein is therefore concluded to be the most important diet constituent controlling 5'D activity.

To identify the source of T_3 saturably bound to nuclear receptors, [^{125}I]T_4 and [^{131}I]T_3 were simultaneously injected (ip) into trout. The percentages of intracellular T_3 saturably bound to nuclear receptors were: gill, 76.2 $\pm$ 5.7%;

kidney, 28.2 ± 4.9%; and liver, 50.5 ± 5.0%. These results indicate that tissues depending on plasma pool T_3 for receptor binding could be more vulnerable to changes in plasma T_3.

It is concluded that changes in hepatic 5'D activity are, at least partially, responsible for changes in plasma T_3 levels. Monodeiodinase activity can be measured in the microsomal fractions of the liver, kidney, muscle and gill; two enzyme systems have been established. The role of intracellular and plasma pool T_3 in supplying T_3 for nuclear receptors in liver kidney and gill differs between the tissues. Together, these results indicate that 5'D is an important factor in regulation of the thyroid hormone system.

ACKNOWLEDGEMENTS

I would like to thank my Committee members, Drs. E. Huebner and D. Fitzpatrick, for their interest and comments over the past few years on my thesis work. In addition, I would also like to thank Dr. H. John-Aalders for agreeing to be my external examiner, and his reading of the thesis.

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To my parents, Cyrus and Ann MacLatchy, and my sisters Laura MacLatchy and Heather MacLatchy-Gaudet, thanks for your continued long-distance encouragement and support, even when you were worried I was turning into a union hack instead of a scientist. Special thanks to Bill Campbell for his support and prodding when I needed it most, as well as all his help

with the production of the thesis.

Last, but certainly not least, I have had the privilege of having Dr. J.G. Eales as my thesis supervisor, and 'thanks' do not cover the gratitude I feel for his input, enthusiasm, and untiring patience. His ability to get enthused about even the smallest finding, and to see something positive in even the largest failure have made it a pleasure to be one of his students. I have benefitted in innumerable ways having had the support of this fine scientist.

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LIST OF ABBREVIATIONS

Abbreviation	
C	Control
^0C	Degrees Celsius
Ci	Curie
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
CHO	Carbohydrate
D	Dark
DTT	Dithiothreitol
E	Experimental
EDTA	Ethylenediamine-tetraacetic acid
E2	17β-estradiol
5'D	5'-Monodeiodinase
g	Gram; X Force of Gravity
GH	Growth Hormone
h	Hour
hGH	Human Growth Hormone
HSI	Hepatosomatic Index
IU	International Unit
ip	Intraperitoneal
K	Kilo-
K_m	Enzyme affinity (Michaelis-Menten Constant)
L	Light; litre
m	Milli-

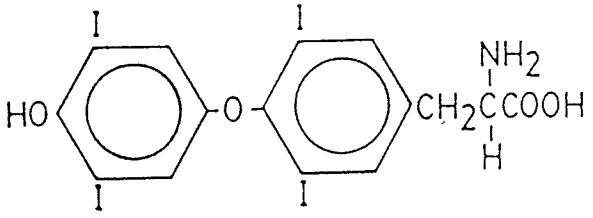
μ	Micro-
M	Molar
min	Minute
MS222	Methane tricaine sulfonate
MT	∝-methyl Testosterone
n	Nano-
pi	Post-injection
prot	Protein
PTU	Propylthiouracil
RBC	Red Blood Cells
rpm	Revolutions/minute
s	Second
sGH	Salmon Growth Hormone
T	Testosterone
T_0	Thyronine
T_3	3,5,3'-triiodo-L-thyronine
T_4	Thyroxine
TP	Testosterone Propionate
TSH	Thyroid Stimulating Hormone
w/v	Weight/Volume
V_{max}	Maximal velocity of enzyme reaction

GENERAL INTRODUCTION

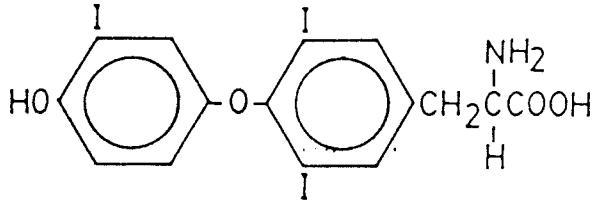
When studies began on the thyroid hormone system, thyroxine (T_4; Figure 1), which is released by the thyroid was believed to be the thyroid hormone. The structure of T_4 was first determined by Harrington and Barger (1927). It was twenty five years later when Gross and Pitt-Rivers (1952) first identified 3,5,3'-triiodo-L-thyronine (T_3; Figure 1) in plasma. The concept that T_4 is the primary thyroid hormone has changed drastically over the last twenty years with the development of assays that have accurately detected the presence of T_3 (Sterling et al, 1969; Brown et al, 1970 and Dussault et al, 1971), discovery of a 5'-monodeiodinase (5'D) enzyme that converts T_4 to T_3 (Schimmel and Utiger, 1977; Visser, 1978 and Shields and Eales, 1986), and discovery of nuclear receptors that have a 10-20X greater affinity for T_3 over T_4 (Oppenheimer, 1983; Oppenheimer, 1986 and Bres and Eales, 1986). It is now the generally accepted view that T_3 is the presumed biologically active thyroid hormone.

Based on the small number of fish studies to date, T_4 is probably the only hormone released by the thyroid. After administration of thyroid stimulating hormone (TSH) there is an increase in thyroidal release of T_4 into the plasma with no concurrent change in plasma T_3 (Chan and Eales, 1975; Brown et al, 1978; Milne and Leatherland, 1978). T_4 then enters the tissues from the blood stream and is deiodinated by 5'D to T_3. Monodeiodination in teleosts has been demonstrated in vivo

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



THYROXINE (T4)



3,5,3'-TRIIODO-L-THYRONINE

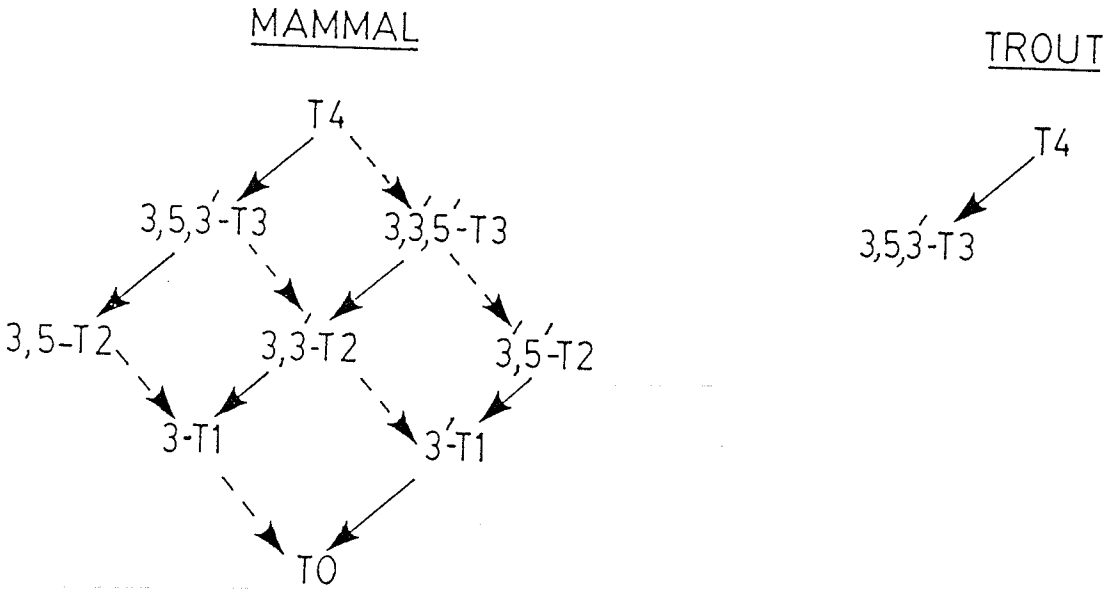
(T3)

(Eales, 1977; Higgs and Eales, 1977; DeLuze and Leloup, 1981; 1982; DeLuze, 1982) and *in vitro* (Law and Eales, 1973; Leatherland, 1981; Pimlott and Eales, 1983; Shields and Eales, 1986). Unlike mammals, neither inner-ring monodeiodination to 3,3',5'-triiodo-L-thyronine (reverse T3; rT_3) has been demonstrated (Eales *et al*, 1983; Shields and Eales, 1986), nor further significant conversion of the original T_4 deiodination product to diiodo- or monoiodo-thyronine, or thyronine (T_2, T_1, T_0). Overviews of the mammalian and teleostean deiodination pathways are shown in Figure 2.

The T_3 which is produced in trout binds to putative nuclear receptors (Van der Kraak and Eales, 1980; Bres and Eales, 1986). In mammals, binding to the nuclear receptor produces changes in mRNA concentrations, which indicate that the hormone-receptor complex acts on chromatin to alter gene expression (Oppenheimer, 1983). It is thought, but without evidence, that this may also be the method by which T_3 acts in teleosts. Since all T_3 produced in teleosts is probably generated from the extrathyroidal monodeiodination of T_4, this conversion is critical in the regulation of thyroid hormone expression.

The common roles of thyroid hormones in the vertebrate classes are their involvement in control of metabolism, growth and development. Thyroid hormones have been described as permissive hormones (Eales, 1985), because they tend to act in conjunction with other hormones and factors, rather than in

Figure 2. Deiodination pathways in the mammal and trout. Conversion of T_4 to T_3 (3,5,3'-T_3) is by 5'D. Deiodination in mammals can continue until all iodines are removed (thyronine; T_0).


MAMMAL
T4
3,5,3′-T3
3,3′,5′-T3
3,5-T2
3,3′-T2
3,5′-T2
3-T1
3′-T1
T0
TROUT
T4
3,5,3′-T3

isolation. There are many factors which have previously been shown, through mainly circumstantial evidence, to have an effect on the deiodination of T_4 to T_3 in teleosts. These factors include nutritional state, temperature, stress and hormones (Eales and MacLatchy, 1989).

Deiodination by 5'D in salmonids has been identified in liver and kidney (Leatherland, 1981; Shields and Eales, 1986). To define the role of T_4 deiodination in determining thyroidal status in teleosts, further elucidation of the 5'D system(s) in teleosts is required. This thesis focuses on characterization of the T_4 5'D system(s) in salmonids, mainly the rainbow trout (Oncorhynchus mykiss), and the determination of whether the influence of various factors (primarily endocrine and nutritional) on the peripheral thyroid hormone system can occur at the level of the 5'D enzyme. In order to achieve these goals, the following was done: (1) determine the tissues in which the 5'D enzyme activity occurs, and the properties of the enzyme(s) involved; (2) examine the effects of the hormones testosterone, estradiol, T_3, and growth hormone on T_4 deiodination; (3) examine the effects of dietary lipid, protein and carbohydrate on 5'D activity; (4) determine whether the nuclear T_3 receptors of certain tissues are occupied by locally generated T_3 or T_3 derived from plasma.

CHAPTER 1

PROPERTIES OF T_4 5'-DEIODINATING SYSTEMS IN VARIOUS TISSUES OF THE RAINBOW TROUT

INTRODUCTION

Two different enzymes that convert T_4 to the physiologically more active T_3 have been characterized in various mammalian tissues. Type I is a high Km (1 uM), thiol-stimulated, propylthiouracil (PTU)-inhibited, 5'D enzyme found in liver, kidney and thyroid and which operates through a ping-pong mechanism (Kaplan and Utiger, 1978; Leonard and Rosenberg, 1978a; 1978b; Visser et al, 1978; Balsam et al, 1979; Laurberg and Noye, 1982). Type II 5'D is a low Km (1 nM) enzyme that is stimulated by thiols, but is not inhibited by PTU, and has a sequential mechanism. It occurs in the central nervous system, brown adipose, placental and pituitary tissues (Silva et al, 1978; Visser et al, 1981, 1982, 1983; Leonard et al, 1983; Kaplan and Shaw, 1984). There are also two avian hepatic 5'D systems with K_m values for T_4 in the micromolar and nanomolar ranges, the latter being unaffected by PTU (McNabb et al, 1986; Hughes and McNabb, 1986).

In poikliotherms, Galton (1988) has identified two 5'D enzymes in the amphibian Rana catesbeiana, with micromolar and nanomolar K_m values; the low K_m system has properties similar to those described for the mammalian Type II 5'D. A 5'D system has also been demonstrated in teleost fish. Shields

and Eales (1986) described a microsomal 5'D enzyme in the liver of rainbow trout. The enzyme had a Km of 1 nM, was stimulated by thiols and was inhibited by PTU. In the same species, Leatherland (1981) reported an enzyme that converted T_4 to T_3 in the kidney and liver.

To further understand the regulation of thyroidal status in the peripheral tissues of teleost fish, it is necessary to determine in which tissues deiodination occurs and the properties of the enzyme systems involved. The present objectives are to determine by in vitro procedures the optimal assay conditions for deiodinating systems in several tissues of the rainbow trout, and to investigate aspects of the underlying kinetic mechanisms.

MATERIALS AND METHODS

Fish Maintenance:

Rainbow trout ranging from 175 to 400 g were obtained from the Rockwood Hatchery, Balmoral, Manitoba, and held on a 12-h L : 12-h D photoperiod in 2.3-kL fiberglass tanks supplied with flowing, dechlorinated, aerated Winnipeg city water at 12°C. Trout were fed once daily with trout grower pellets (Martin Feed Mills, Ontario) at a ration of 1 - 2 % per day.

Sampling Procedure:

Trout were anaesthetized (0.1 g/L methane tricaine sulfonate; MS222) between 0830 h and 0930 h, and then killed

by concussion and the liver, kidney, gill and/or skeletal muscle tissue removed. Blood was removed from the caudal vessels of anaesthetized fish using a heparinized syringe, and red blood cells (RBC) isolated by centrifugation at 17,000 g for 3 min. White muscle tissue was used exclusively and was taken from the region between the dorsal fin and the dark muscle at the mid-line of the body wall.

Tissues were rinsed with ice-cold buffer [0.1 M KH_2PO_4/Na_2HPO_4, 0.25 M sucrose, 3 mM dithiothreitol (DTT), 5 mM EDTA]. Tissues were pooled from several (4-8) fish. The tissues were blotted dry, weighed and minced with scissors in 4 volumes (weight/volume; w/v) of buffer. Tissues were dispensed into 55-mL Wheaton homogenizer chambers. Liver and kidney were then homogenized in a Polytron (Brinkman Instruments; setting 6 for 4 s) followed by two strokes with a motorized pestle (Tri-R Instruments Inc, New York). The resulting crude homogenate was filtered through two layers of cheesecloth. The final homogenate to buffer ratio was adjusted to 1:8 (w/v). Gill filaments were homogenized for 15 s in the Polytron in four volumes of buffer. Muscle tissue was filtered through 8 layers of cheesecloth. RBC were homogenized by two strokes with the motorized pestle. Otherwise all homogenization procedures were similar to that used for liver.

Aliquots of homogenate were transferred into centrifuge tubes and spun for 20 min at 730 g in a Ti50 fixed-angle rotor

(Beckman Instruments, Palo Alto, California). The resulting supernatant was spun at 25,200 g for 20 min to obtain the crude mitochondrial/lysosome pellet. The supernatant was then spun at 110,000 g for 1 h to separate the microsome pellet from the cell sap. The pelleted fraction was then resuspended in 4 - 5 mL of buffer and stored at -70°C for up to 3 months.

T_4 5'D Assay:

The assay was based on the procedure of Shields and Eales (1986). Thawed fractions were diluted to the appropriate protein concentration (approx. 5 mg/mL in final assay volume) with buffer to a volume of 1 mL. Initially, pH and DTT concentrations were varied to find the assay optima for each tissue. Three replicate tubes were equilibrated for 30 min in darkness in a waterbath (12°C, 150 rpm) and T_4 substrate was then added. The T_4 substrate, dissolved in 20 µL 0.1 M NaOH, consisted of approximately 100 000 cpm of [^{125}I]T_4 labelled in the phenyl ring (specific activity 1200 uCi / ug; $^{125}I^-$ content between 8 -16 %; Amersham International) and carrier T_4 (Sigma, St. Louis) which was varied to provide a range of final substrate concentrations.

After 30 min of incubation with T_4 substrate, 20 µl of KI (2×10^{-3} M) were added to each assay tube and duplicate 100-µL aliquots of the reaction medium pipetted onto Sephadex G-25 mini-columns containing 100 µL of 0.1N NaOH for $^{125}I^-$ determination. $^{125}I^-$ was separated from labelled organic compounds by collecting the second of two 2.5-mL washes of

barbital buffer (pH 8.6). Parallel control tubes were incubated in each assay. These contained buffer plus any reagents added to the experimental tubes, but no subcellular fractions. The $^{125}I^-$ level in these control tubes was subtracted from that in the corresponding assay tubes to determine the enzymatically generated $^{125}I^-$. The T_4 deiodination rate (pmol T_4 converted/ h / mg protein) was calculated as the product of the fraction of the added [^{125}I]T_4 deiodinated to $^{125}I^-$ and the total T_4 added to the incubation tube, in relation to incubation time and protein concentration (Appendix 1). The final protein concentration in each incubate was determined by the Bio-Rad protein method (Bradford, 1976). Lineweaver-Burk plots were calculated by the least-squares method to determine Vmax and Km values.

RESULTS

Determination of pH optima at two substrate concentrations:

Deiodinase activity was examined at a DTT concentration of 5 mM at pH values of 5, 6, 7, 8 and 9 at T_4 concentrations of 1 nM (low substrate) and 10 nM (high substrate). At low substrate, muscle, gill and liver exhibited a single pH optimum at 7 (Figure 3) with no activity detectable in kidney or RBC. At high substrate, the pH optimum for liver was 7, but for kidney two pH optima at 6 and 8 were observed (Figure 4), with no activity detectable in gill, muscle or RBC.

Determination of optimal DTT concentration:

Figure 3. The influence of pH on 5'D at low substrate concentration (1 nM) for microsomal liver (L), muscle (M) and gill(G) for two separate pools.

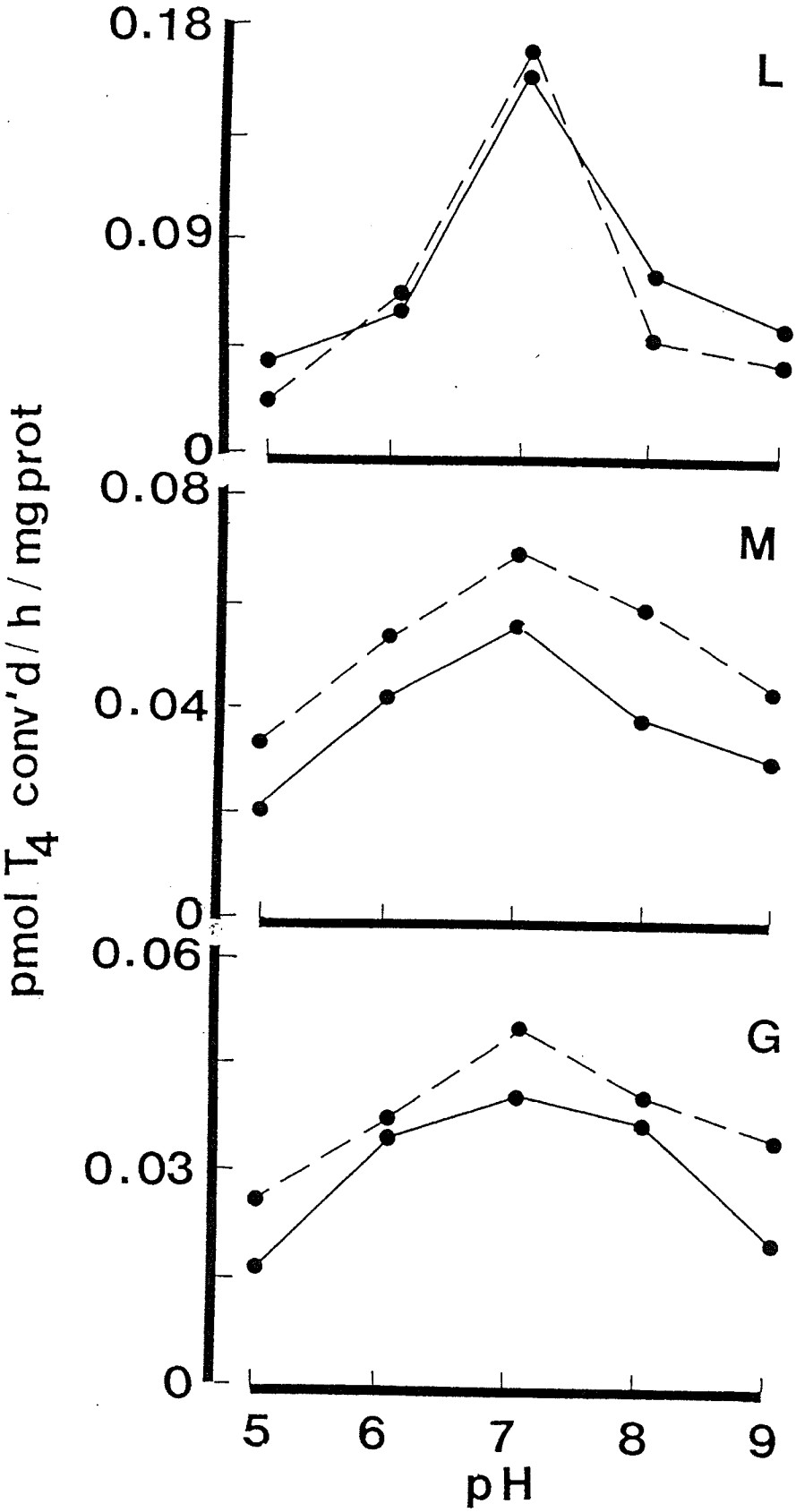
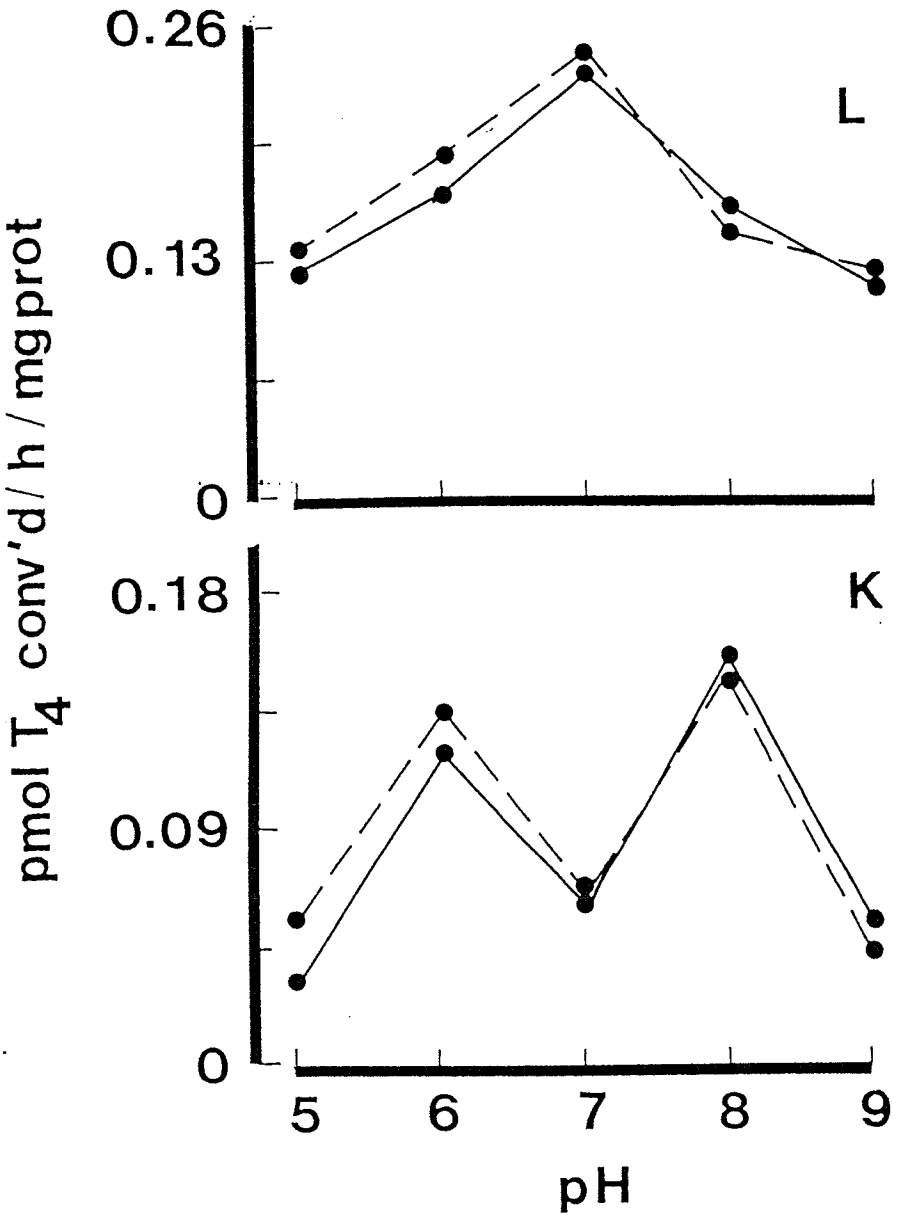

0.18
0.09
0
L
0.08
0.04
0
M
0.06
0.03
0
G
pmol T_4 conv'd / h / mg prot
5
6
7
8
9
pH

Figure 4. The influence of pH on 5'D at high substrate concentration (10 nM) for liver (L)and kidney (K)for two separate microsomal pools.


0.26
0.13
0
L
0.18
0.09
0
K
pmol T_4 conv'd / h / mg prot
5
6
7
8
9
pH

Microsomes were resuspended in buffer at optimum pH (pH 6 for kidney and pH 7 for the other tissues). The DTT concentration was varied from 2 to 14 (low substrate) or 22 mM (high substrate). At the low T_4 concentration, liver 5'D activity increased between 2 and 10 mM DTT with no change in activity between 10 and 14 mM DTT (Figure 5). Maximal 5'D activity occurred at 8 mM DTT in gill, and at 7 mM DTT in muscle (Figure 5). At the high substrate concentration, 5'D activity was maximal at 15 mM DTT for both liver and kidney (Figure 6). RBC showed no 5'D activity at any DTT concentration assayed at low or high T_4 concentrations at pH 6, 7 or 8. Kidney at low T_4 substrate concentration and gill and muscle at high T_4 concentration had no enzyme activity between pH 6 and 8 over the DTT range of 2-22 mM.

Determination of K_m:

Liver, kidney, gill and muscle 5'D activities were measured at optimum pH and DTT concentration over the low substrate range of 0.08 to 1.3 nM T_4 and over the high substrate range of 1.6 to 25 nM T_4. The Vmax and Km were determined from two trials for each substrate range (Table 1). At low substrate, the mean Km for liver was 1.0×10^{-10} M, the mean Km for gill was 2.0×10^{-10} M and the mean Km for muscle was 1.7×10^{-10} M. At high T_4 substrate, liver and kidney had mean Km values of 1.0×10^{-8} M and 1.5×10^{-8} M respectively.

Influence of DTT:

DTT at 25%, 50% and 100% of optimum concentration was

Figure 5. The effect of DTT on 5'D at low substrate concentration (1 nM) for liver (L), muscle (M) and gill (G) for two separate microsomal pools.

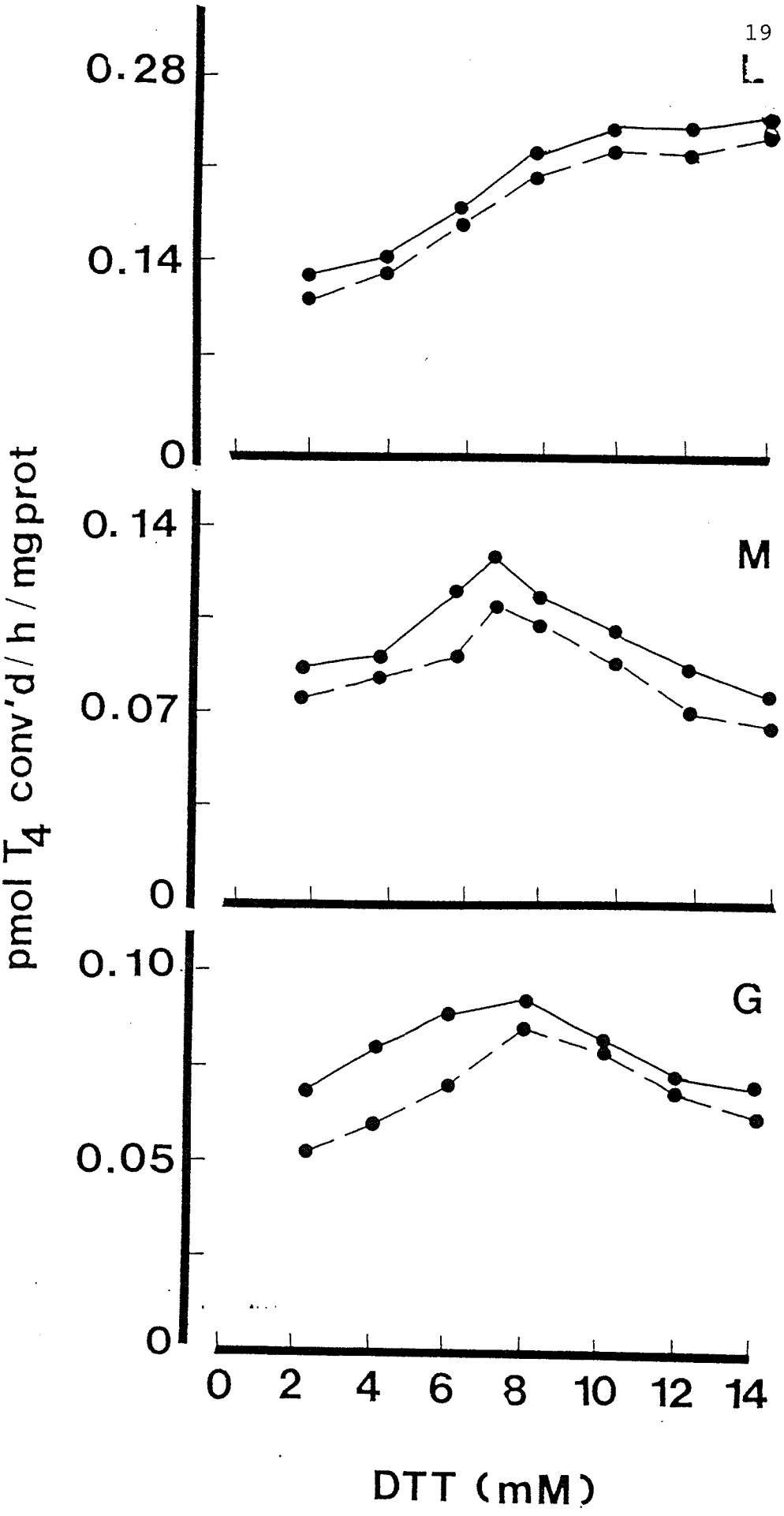

0.28
0.14
0
L
0.14
0.07
0
M
0.10
0.05
0
G
pmol T_4 conv'd / h / mg prot
0 2 4 6 8 10 12 14
DTT (mM)

Figure 6. The effect of DTT on 5'D at high substrate concentration (10 nM) for liver (L), and kidney (K) for two separate microsomal pools.

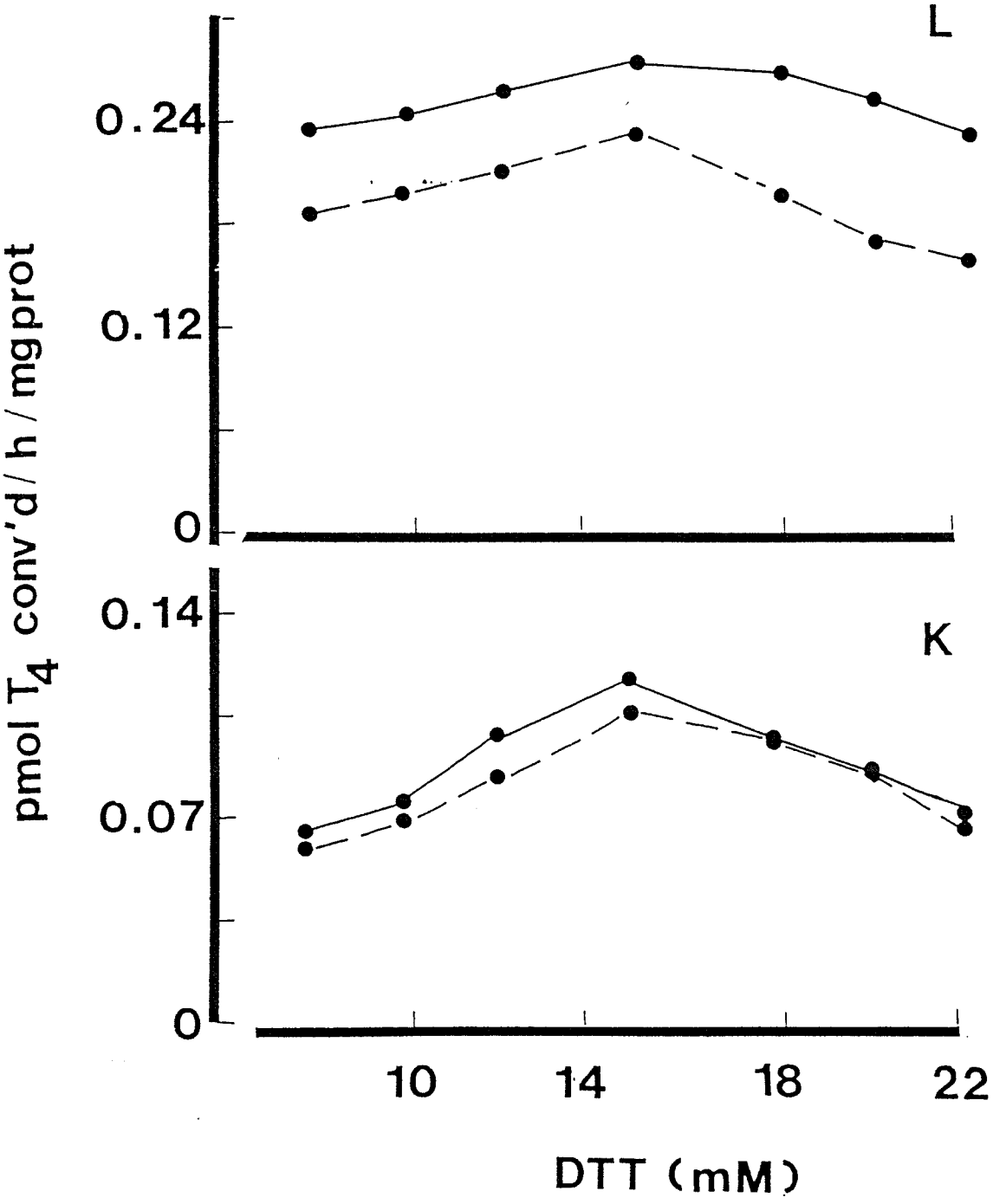
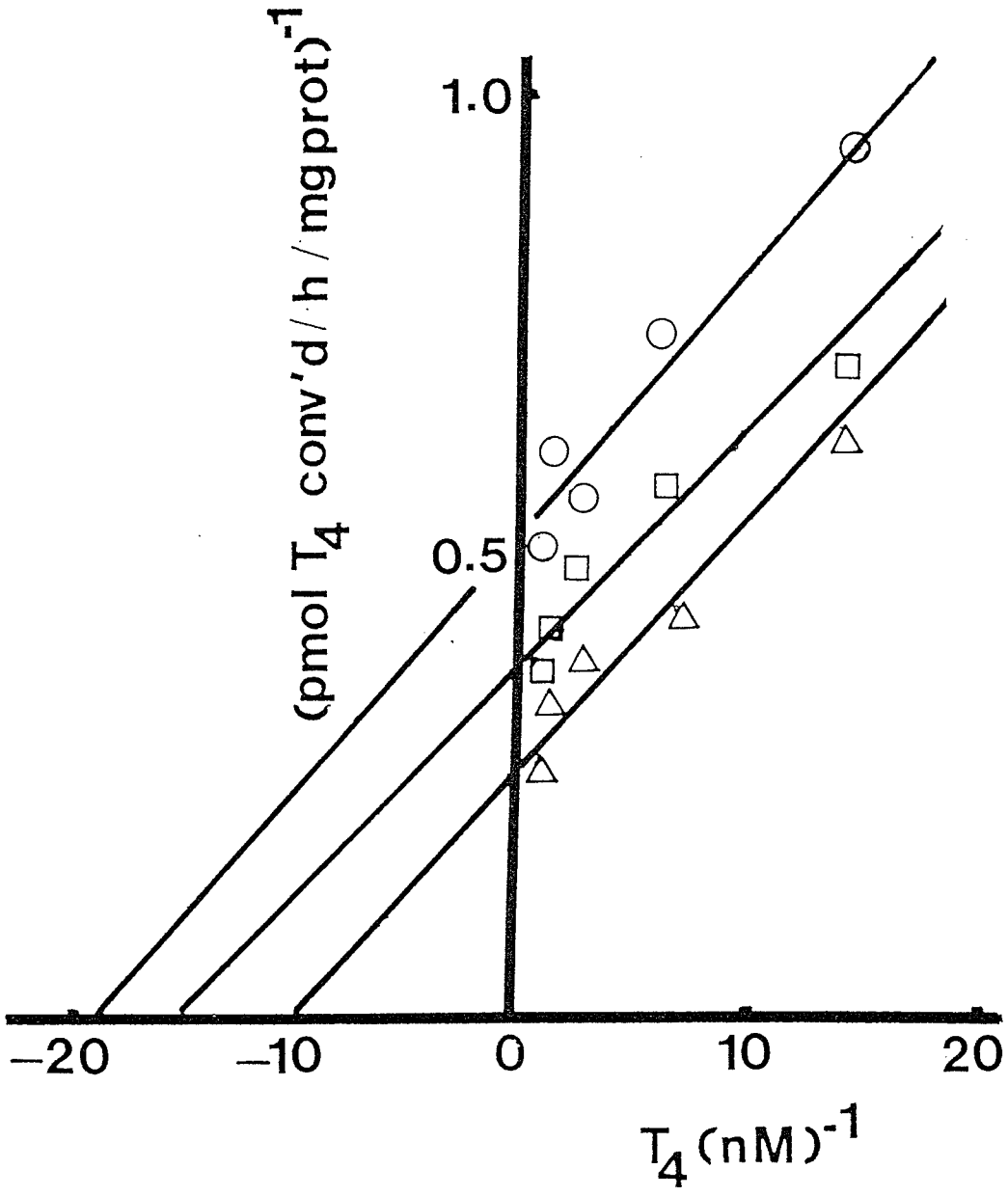

L
K
0.24
0.12
0
0.14
0.07
0
pmol T_4 conv'd / h / mg prot
10
14
18
22
DTT (mM)

Table 1. Mean K_m and V_{max} values for 5'D in microsomal fractions of rainbow trout tissues. Values are the $\bar{X} \pm$ S.E.M. from trials based on two separate pools.

Tissue	T_4 Substrate Concentration (nM)	K_m (M)	V_{max} (pmol conv'd/ h/mg prot)
Liver	0.08 to 1.3	$0.98 \times 10^{-10} \pm 6.1 \times 10^{-13}$	3.74 ± 0.05
Muscle	0.08 to 1.3	$1.98 \times 10^{-10} \pm 4.5 \times 10^{-13}$	0.794 ± 0.0042
Gill	0.08 to 1.3	$1.68 \times 10^{-10} \pm 3.1 \times 10^{-13}$	0.624 ± 0.0037
Liver	1.6 to 25	$1.00 \times 10^{-8} \pm 7.6 \times 10^{-11}$	8.21 ± 0.22
Kidney	1.6 to 25	$1.47 \times 10^{-8} \pm 6.2 \times 10^{-11}$	5.76 ± 0.09

Figure 7. Lineweaver-Burk plot of DTT stimulation of hepatic microsomal 5'D from the same sample pool (n=5). Concentrations of DTT added to incubate were 10 mM (△), 5 mM (□) and 2.5 mM (○).


(pmol T_4 conv'd / h / mg prot)$^{-1}$
1.0
0.5
−20
−10
0
10
20
T_4 (nM)$^{-1}$

used over the low and high substrate ranges for which Km values were previously determined. DTT affected the velocity and K_m of the deiodination reaction over the low substrate range for liver (Figure 7), muscle (Figure 8) and gill (Figure 9), and affected only the velocity over the high substrate range for liver (Figure 10) and kidney (Figure 11).

Influence of PTU:

PTU (0, 1 and 10 mM) was added to incubates containing optimum DTT levels. PTU inhibited the action of 5'D at low substrate concentrations for liver (Figure 12), gill (Figure 13) and muscle (Figure 14), and had no effect on enzyme activity of liver (Figure 15) and kidney (Figure 16) at high substrate concentrations.

DISCUSSION

This study demonstrates that 5'D activity occurs in liver, kidney, muscle and gill, but not RBC tissue of rainbow trout. The assay conditions necessary to detect 5'D vary between the tissues, and there appear to be two types of systems operating, at high (1.6-25 nM) and low (0.08-1.3 nM) T_4 substrate levels.

Investigations to date on 5'D and conversion of T_4 to T_3 in peripheral tissues in fish have focused primarily on the hepatic site (Eales, 1985; MacLatchy and Eales, 1988, 1990; Vijayan et al, 1988). In mammals and birds, the liver and kidney are the tissues mainly responsible for peripheral conversion of T_4 to T_3 (Chopra et al, 1978; Engler and Burger,

Figure 8. Lineweaver-Burk plot of DTT stimulation of muscle microsomal 5'D from the same sample pool (n=5). Concentrations of DTT added to incubate were 7 mM (△), 3.5 mM (□) and 1.75 mM (○).

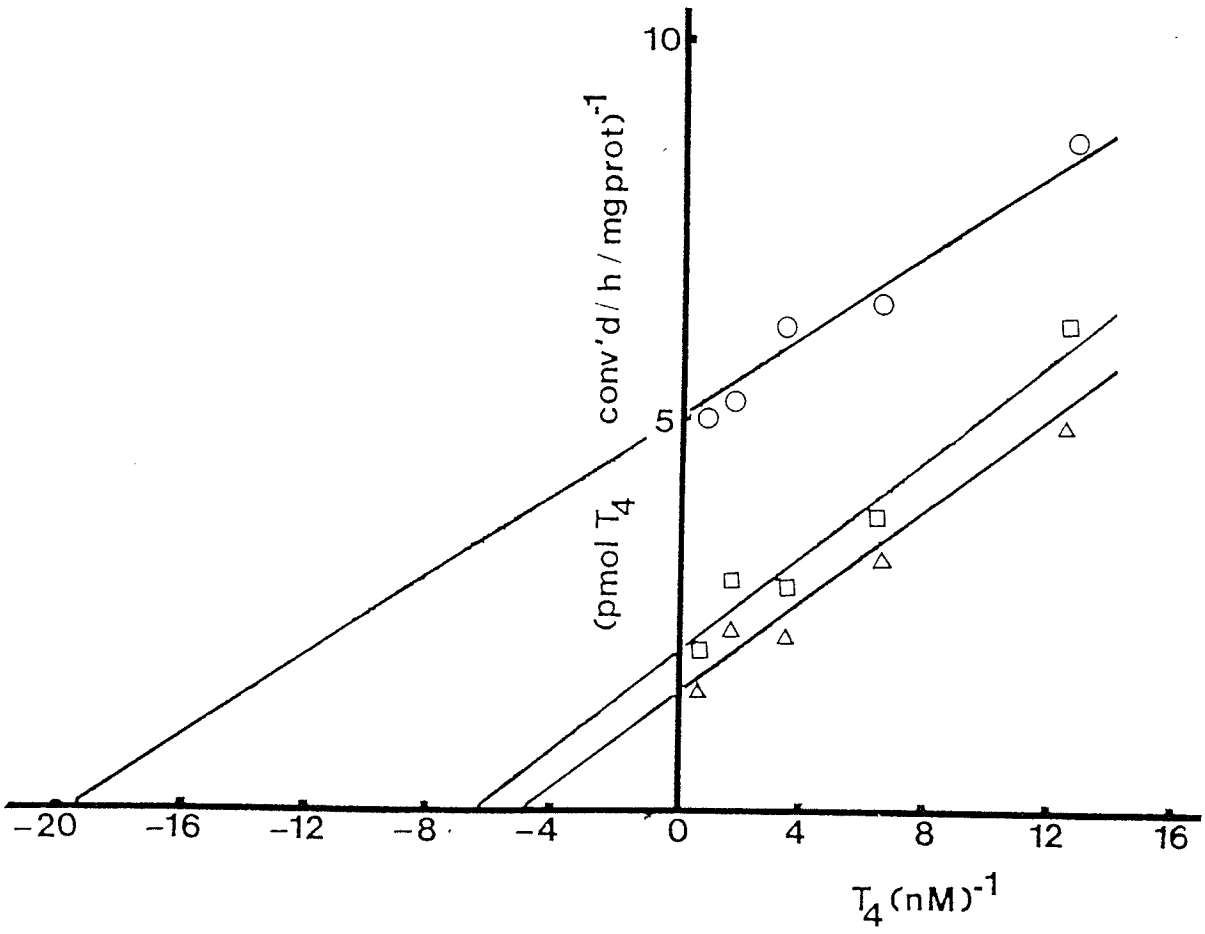

10
5
conv'd / h / mg prot)$^{-1}$
(pmol T_4
−20
−16
−12
−8
−4
0
4
8
12
16
T_4 (nM)$^{-1}$

Figure 9. Lineweaver-Burk plot of DTT stimulation of gill microsomal 5'D from the same sample pool (n=5). Concentrations of DTT added to incubate were 8 mM (△), 4 mM (□) and 2 mM (○).

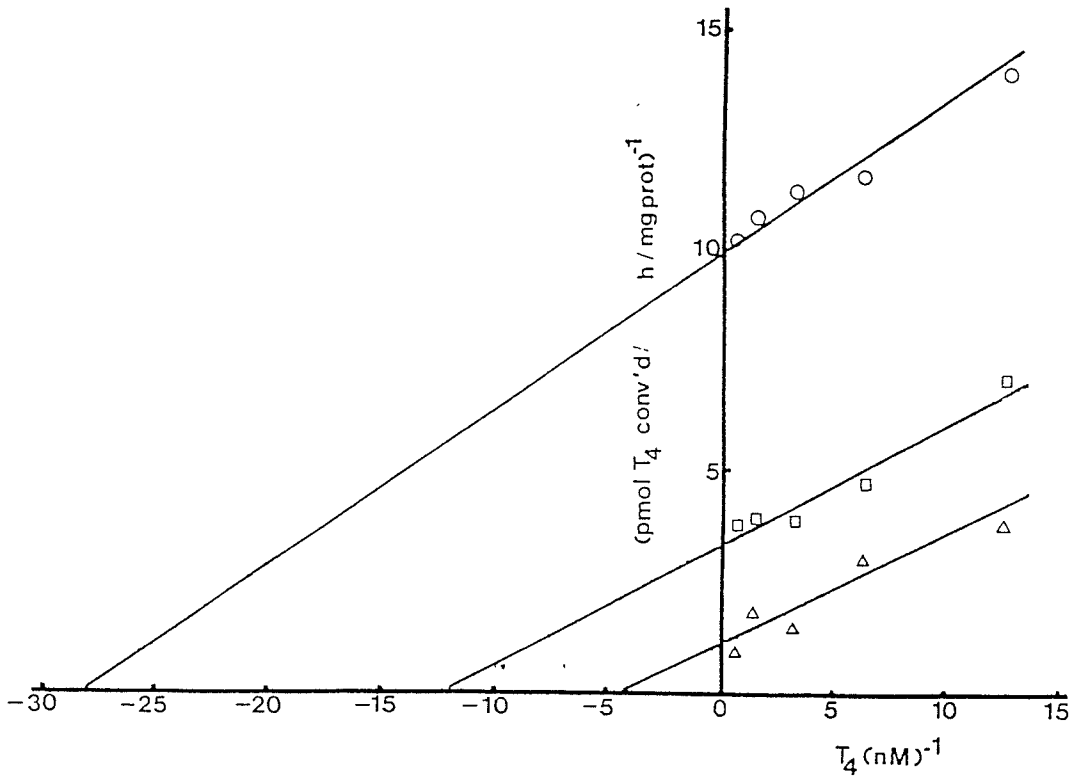

15
10
5
(pmol T_4 conv'd/ h / mg prot)$^{-1}$
−30
−25
−20
−15
−10
−5
0
5
10
15
T_4 (nM)$^{-1}$

Figure 10. Lineweaver-Burk plot of DTT stimulation of hepatic microsomal 5'D from the same sample pool (n=5). Concentrations of DTT added to incubate were 15 mM (△), 7.5 mM (□) and 3.75 mM (○).

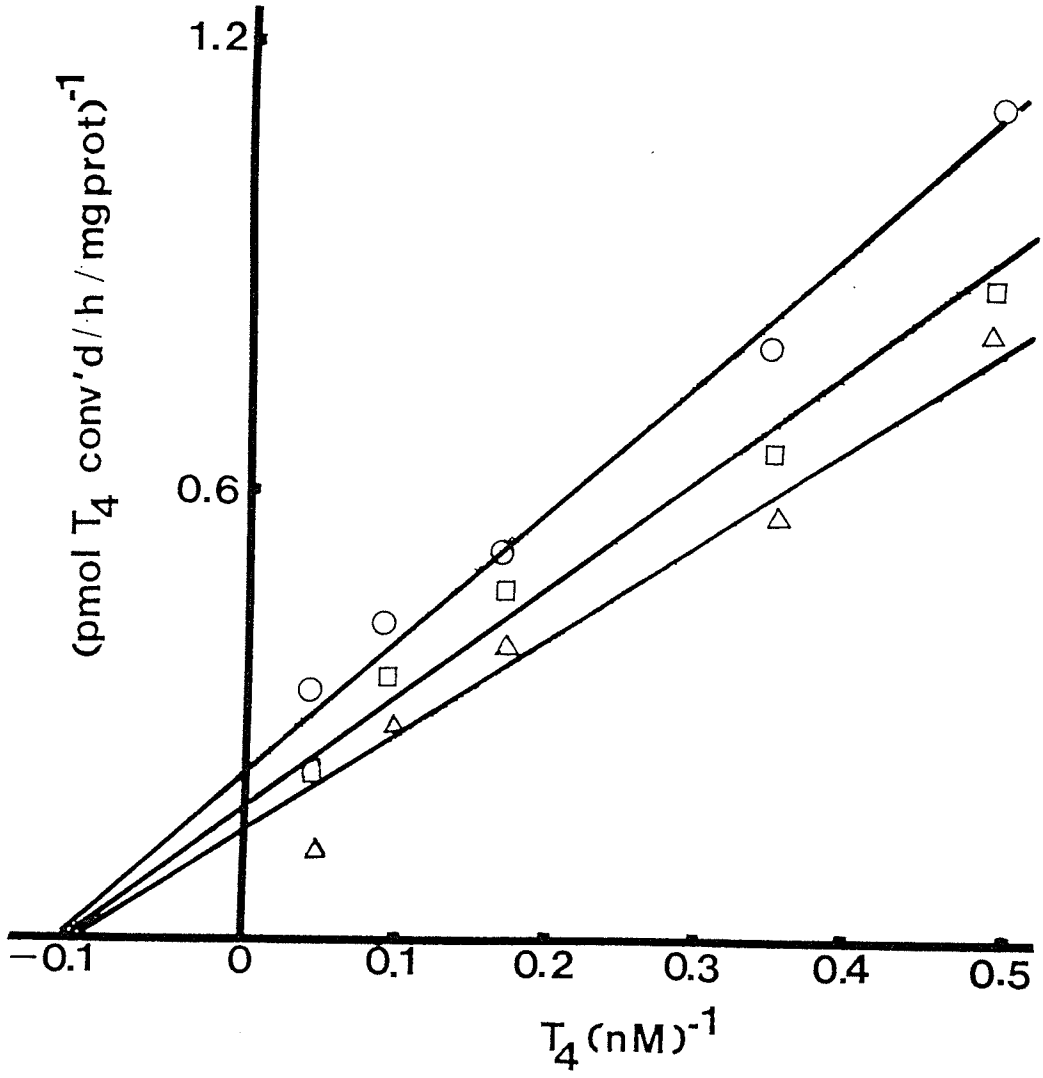

1.2
0.6
(pmol T_4 conv'd / h / mg prot)$^{-1}$
−0.1
0
0.1
0.2
0.3
0.4
0.5
T_4 (nM)$^{-1}$

Figure 11. Lineweaver-Burk plot of DTT stimulation of renal microsomal 5'D from the same sample pool (n=5). Concentrations of DTT added to incubate were 15 mM (△), 7.5 mM (□) and 3.75 mM (○).

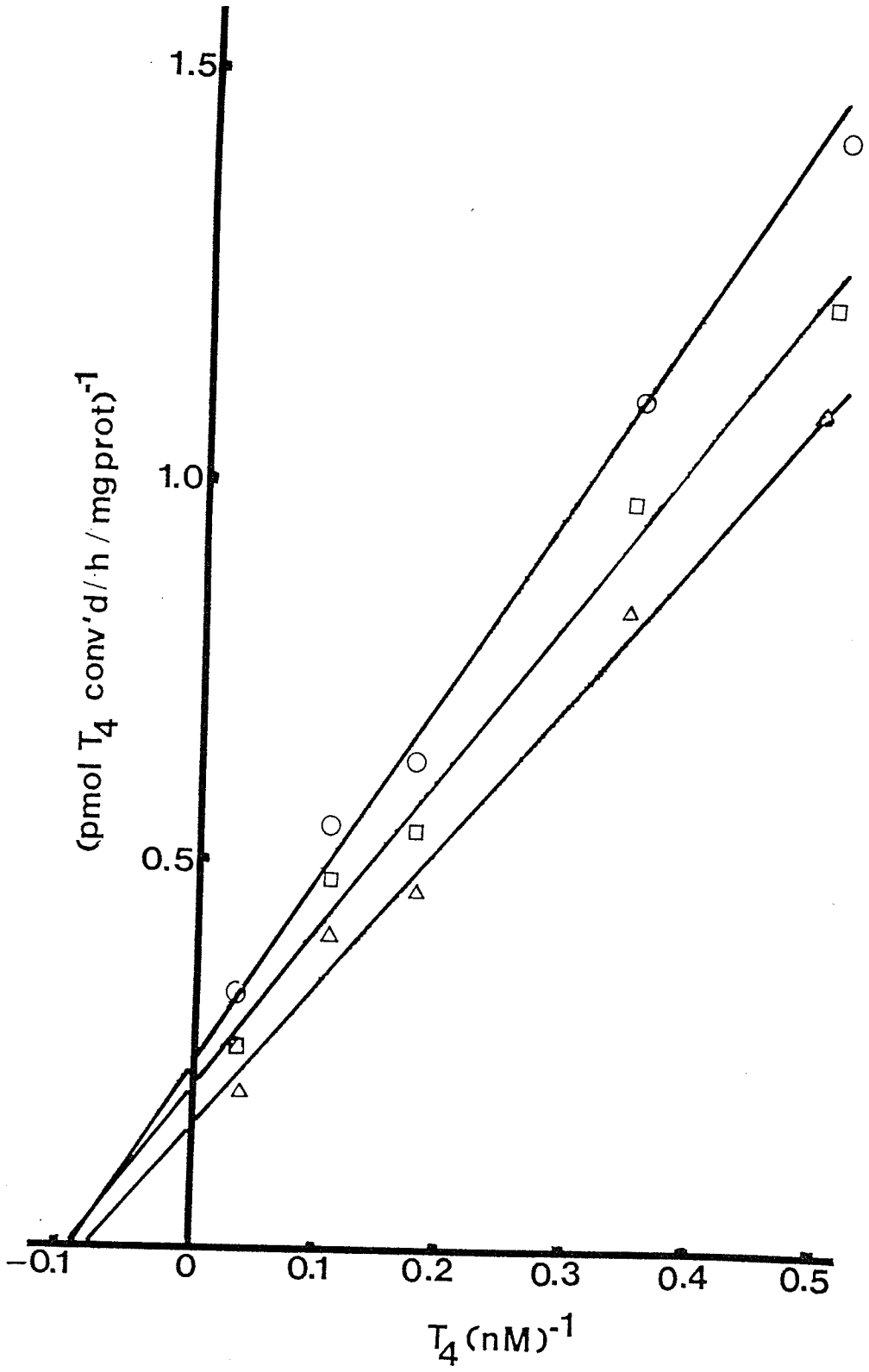

1.5
1.0
0.5
(pmol T_4 conv'd/h/mg prot)$^{-1}$
−0.1
0
0.1
0.2
0.3
0.4
0.5
T_4 (nM)$^{-1}$

Figure 12. Lineweaver-Burk plot of PTU inhibition of hepatic microsomal 5'D from the same sample pool (n=5). Concentrations of PTU added to incubate were 0 mM (▲), 1 mM (■) and 10 mM (●).

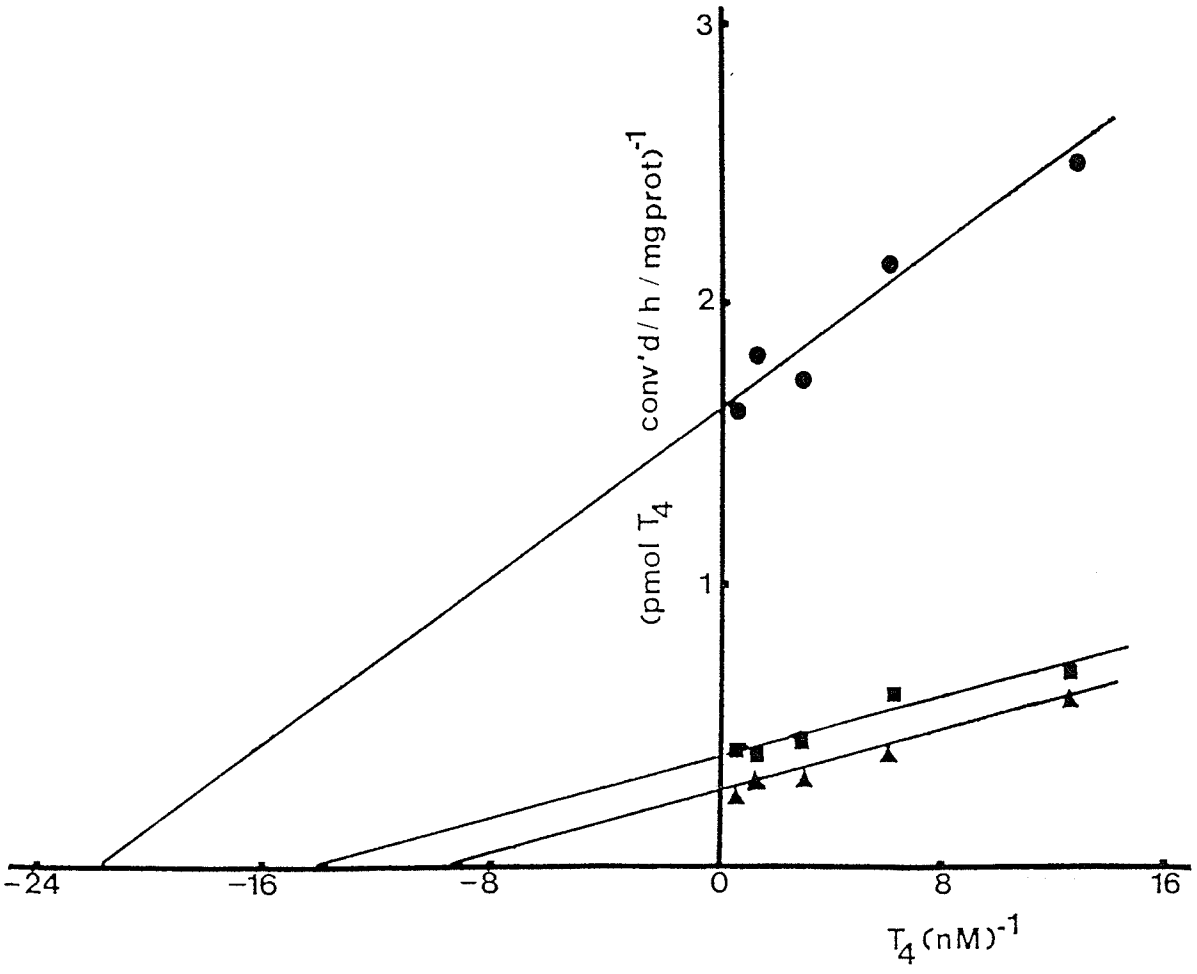

3
2
1
conv'd / h / mg prot)$^{-1}$
(pmol T_4
−24
−16
−8
0
8
16
T_4 (nM)$^{-1}$

Figure 13. Lineweaver-Burk plot of PTU inhibition of gill microsomal 5'D from the same sample pool (n=5). Concentrations of PTU added to incubate were 0 mM (▲), 1 mM (■) and 10 mM (●).

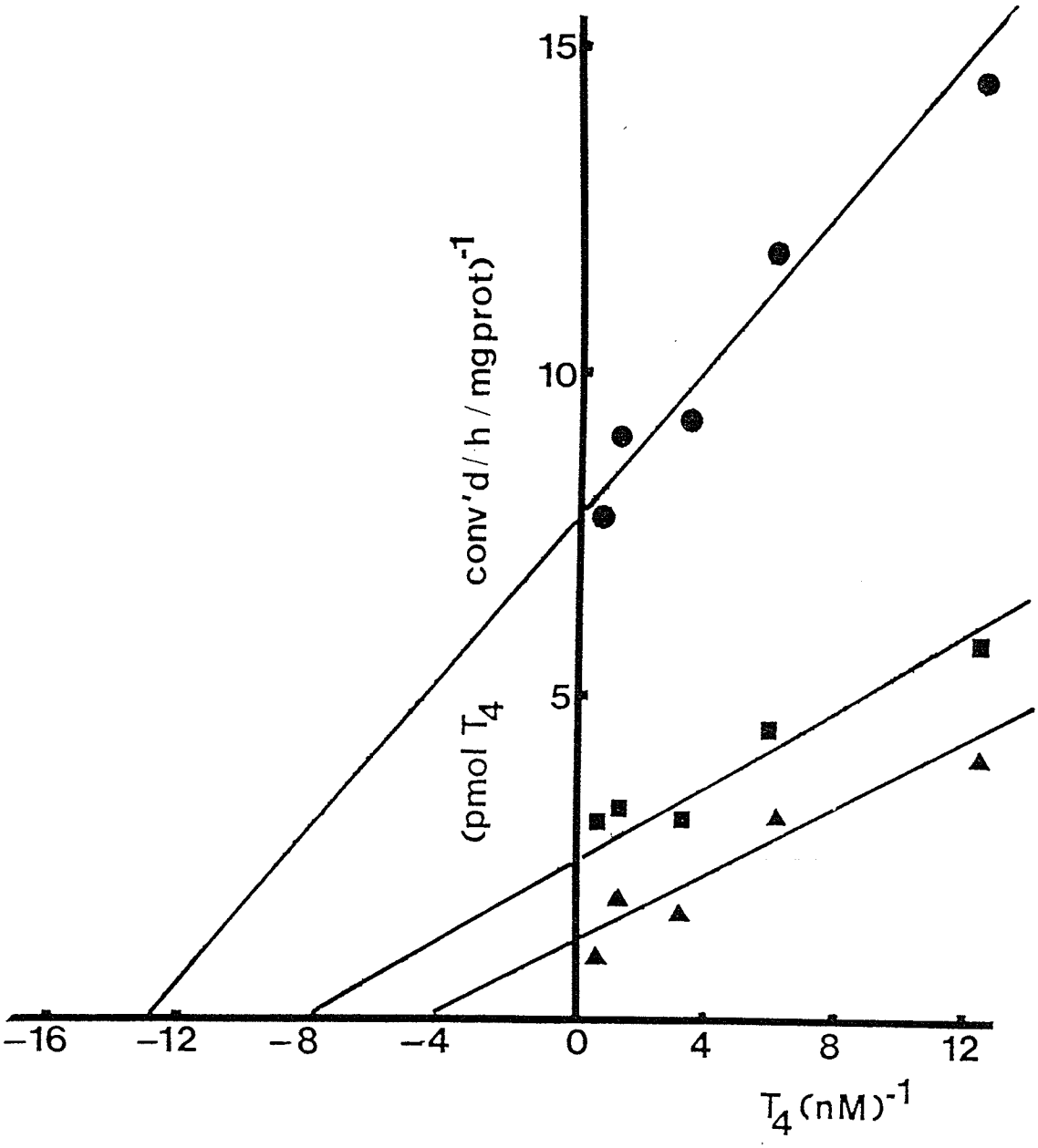

15
10
5
conv'd / h / mg prot)$^{-1}$
(pmol T_4
−16
−12
−8
−4
0
4
8
12
T_4 (nM)$^{-1}$

Figure 14. Lineweaver-Burk plot of PTU inhibition of muscle microsomal 5'D from the same sample pool (n=5). Concentrations of PTU added to incubate were 0 mM (▲), 1 mM (■) and 10 mM (●).

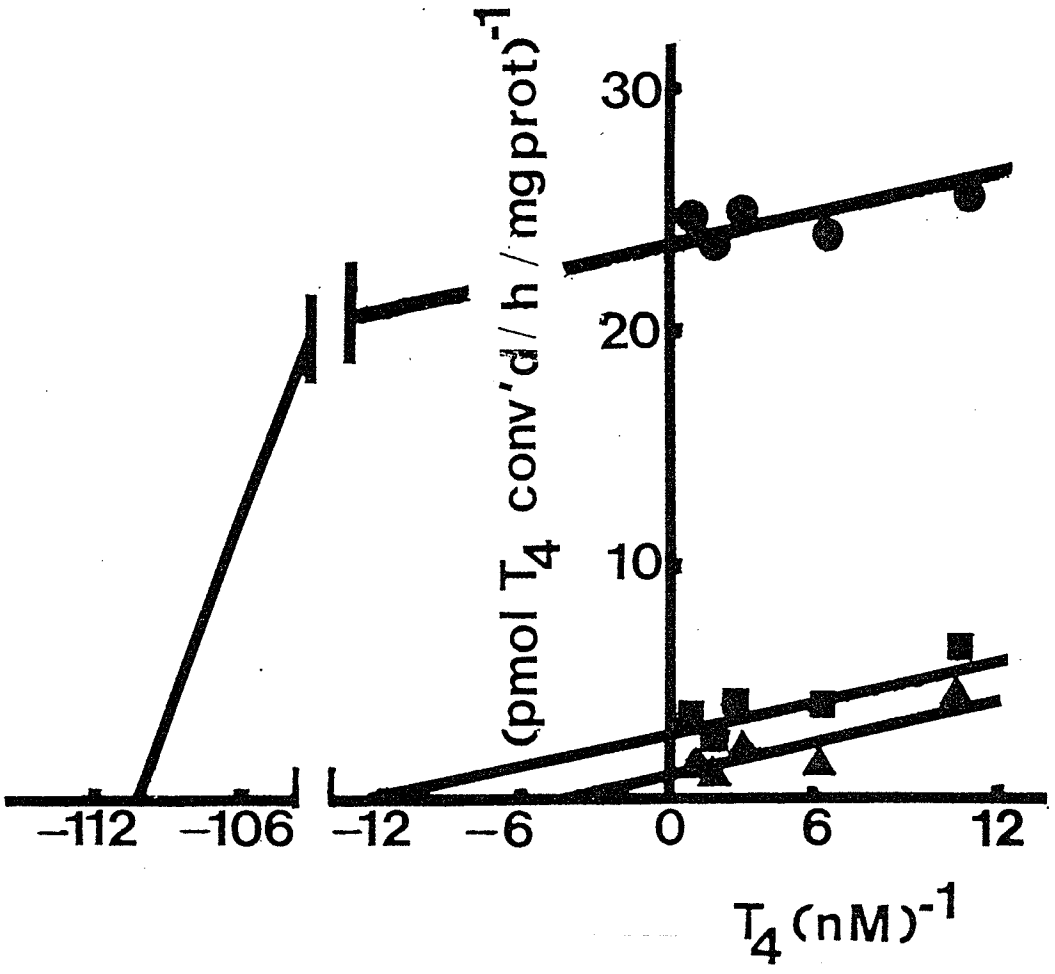

(pmol T4 conv'd / h / mg prot)-1
30
20
10
-112
-106
-12
-6
0
6
12
T4 (nM)-1

Figure 15. Lineweaver-Burk plot of PTU inhibition of hepatic microsomal 5'D from the same sample pool (n=5). Concentrations of PTU added to incubate were 0 mM (▲), 1 mM (■) and 10 mM (●).

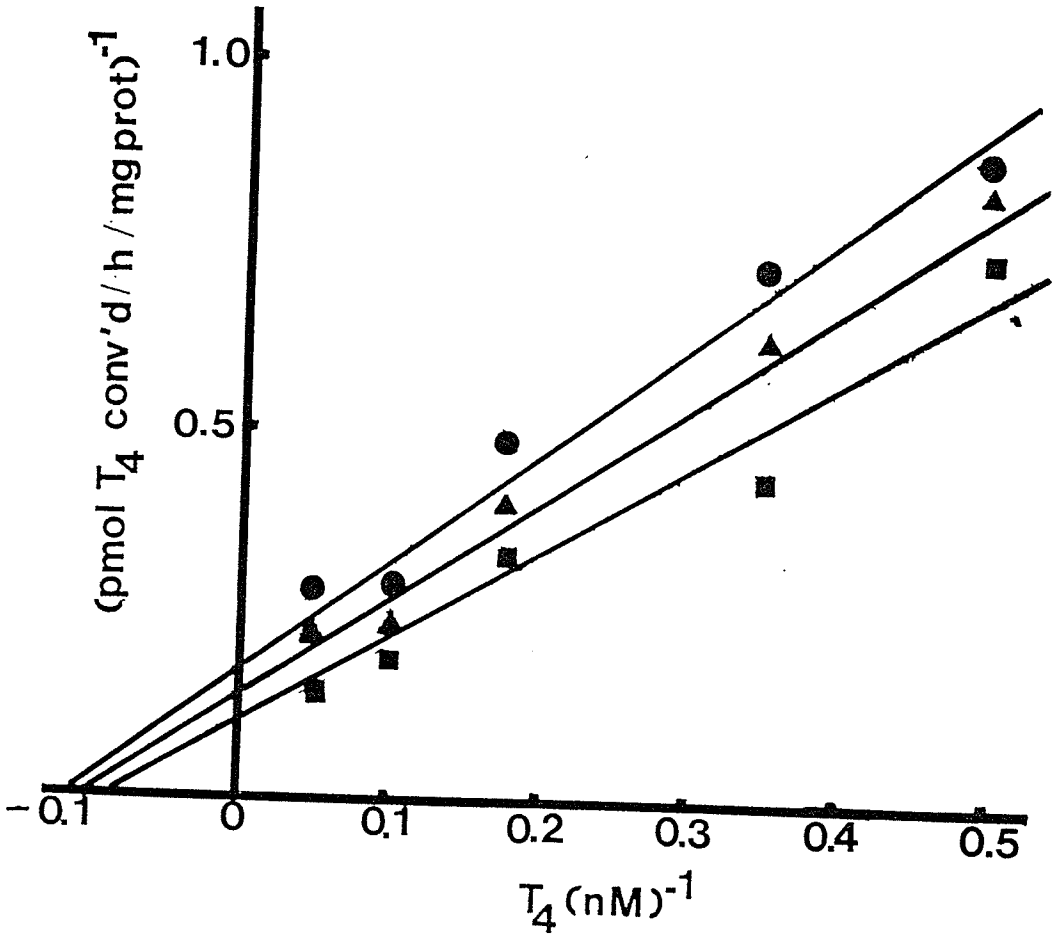
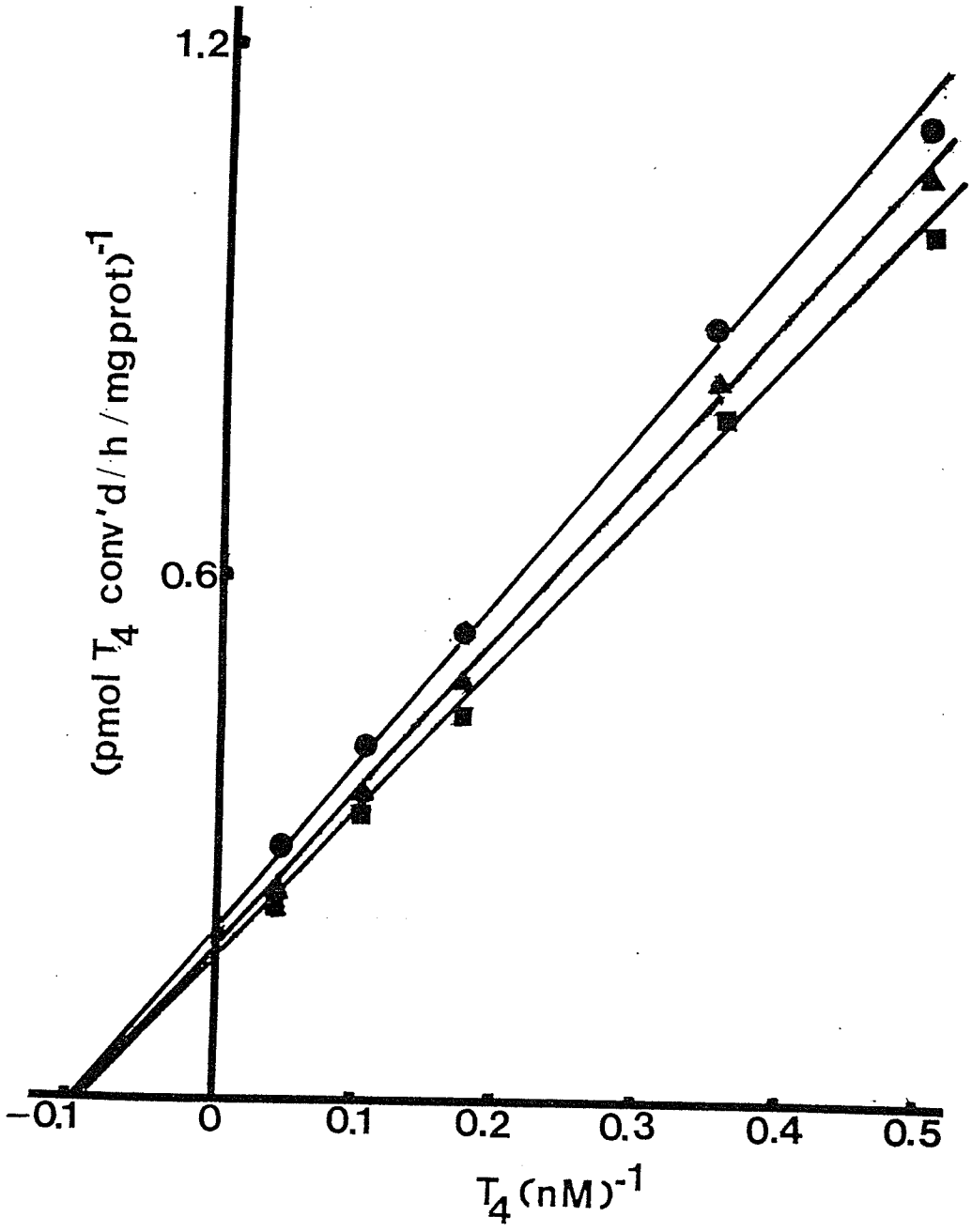

(pmol T_4 conv'd / h / mg prot)$^{-1}$
1.0
0.5
-0.1
0
0.1
0.2
0.3
0.4
0.5
T_4 (nM)$^{-1}$

Figure 16. Lineweaver-Burk plot of PTU inhibition of renal microsomal 5'D from the same sample pool (n=5). Concentrations of PTU added to incubate were 0 mM (▲), 1 mM (■) and 10 mM (●).


1.2
0.6
(pmol T_4 conv'd/h/mgprot)$^{-1}$
−0.1
0
0.1
0.2
0.3
0.4
0.5
T_4 (nM)$^{-1}$

1984; Rudas, 1986; McNabb, 1988), but this may not be so in poikliotherms. In the frog (R. catesbeiana) 5'D has been demonstrated in skin, gut and tail tissue at various life stages, with little or no activity in liver, kidney, heart, brain or muscle of adult frogs (Galton and Hiebert, 1988; Galton, 1988). Thus, focusing solely on hepatic deiodinase may not provide a representative picture of the 5'D system in fish.

In a previous study on rainbow trout in this laboratory (Shields and Eales, 1986), 5'D activity with a K_m of 1 nM was demonstrated in the hepatic microsome fraction. It now appears that there are two microsomal 5'D systems in trout liver. These two systems can be distinguished under different substrate conditions. The low substrate enzyme, which corresponds to that discussed by Shields and Eales (1986) has a pH optimum of 7, and a DTT maximum of 10 mM (this study) and 12 mM (Shields and Eales, 1986), with no change in activity beyond this DTT concentration. On the other hand, the high substrate 5'D system demonstrated in the present study in liver has a pH optimum of 7, and a DTT optimum of 15 mM. Leatherland (1981) also detected a high substrate 5'D enzyme in rainbow trout liver homogenates with a pH optimum of 7. The low substrate 5'D operates at substrate levels which are probably in the physiological range. The high substrate enzyme may be a form of the enzyme that is active only when there are high T_4 levels, such as prior to smoltification

(Dickhoff et al, 1982) or following ingestion of a T_4-rich diet. Alternately, the substrate for the enzyme may not be T_4, but perhaps a T_4 or T_3-sulfate conjugate that is formed for the removal of excess thyroid hormones (Otten et al, 1983). No chromatography was done to test this hypothesis. It is known, however, that T_3 is the only deiodination product of the low substrate enzyme in rainbow trout liver (Shields and Eales, 1986; Sweeting and Eales, unpublished).

Deiodination activity has been shown here in teleost gill tissue for the first time. The 5'D activity in gill tissue has a pH optimum of 7, and a DTT optimum of 8 mM, corresponding closely to the low substrate deiodinating conditions for liver. The possible role of gill deiodinase may relate to the importance of T_3 in the maintainance of Na^+K^+-ATPase activity in teleost gills. A correlation between ATPase activity and plasma T_3 has been shown in salmonids (Folmar and Dickhoff, 1981), and has been conclusively shown in mammals (Ismail-Beigi, 1988). Gill deiodinase could ensure a supply of T_3 in gill tissue even when plasma T_3 is low.

In muscle at low substrate levels the pH optimum for 5'D activity was 7, with a DTT optimum of 7 mM. The 5'D specific activity (V_{max}) present in muscle (0.794 pmol T_4 converted/ h/ mg protein), although low when compared to liver (3.74 pmol T_4 converted/ h/ mg protein), may contribute a significant amount of T_3 to either muscle tissue or plasma T_3. In rainbow trout, muscle represents approximately 67% of total body mass, and

contains about 80% of carcass T_3 (Fok et al, 1990).

Kidney tissue deiodinates T_4 only at high substrate levels, with pH optima of 6 and 8, and an optimum DTT concentration of 15 mM. Leatherland (1981), also employing high substrate levels, demonstrated pH optima in kidney homogenate for 5'D at pH 6 and 9. The two pH optima in kidney may indicate two enzyme systems, or may reflect pH effects on T_4 or T_3 binding to tissue proteins (Leatherland, 1981). The present study did not examine the enzyme kinetics of kidney tissue at the two pH optima to determine the possibility of two enzyme systems. The differences may reflect the role of the kidney in regulating the internal pH of the animal, and the exposure of kidney tissue to a pH range greater than that of the other tissues. It is also possible that different enzymes could occur in different parts of the kidney. In this study, the whole organ was used to determine the presence of 5'D in trout kidney.

No 5'D activity could be demonstrated in RBC under any of the assay conditions. This suggests that T_3, which has been shown to bind to trout RBC nuclear T_3 receptors (Sullivan et al, 1987; Bres and Eales, 1988) is gained solely from the plasma T_3 pool. It also indicates that contamination of other tissues with RBC under the assay conditions used here would not have affected 5'D activity greatly, except by increasing microsomal protein without contributing to 5'D activity.

The amount of functional enzyme (V_{max}) and the affinity of

the enzyme for its substrate (K_m) were measured for each tissue at optimal DTT concentration and optimal pH. The greater K_m values for the high substrate 5'D indicate that much higher levels of substrate must be present to approach 50% of maximum saturation of the enzyme (V_{max}). The low K_m and high V_{max} values for the low substrate hepatic 5'D enzyme suggest that liver is an important site of 5'D activity. The affinity for T_4 is greater than for muscle and gill, and the conversion rate of T_4 is 4.7-6 times greater than for muscle and gill.

One of the main purposes of this study was to determine the kinetic mechanisms of 5'D action, and to compare the results with mammalian, avian and amphibian data. Considerable information exists on the properties of mammalian 5'D systems (see Introduction, Chapter 1 and Appendix 2).

Sulfhydryl groups at the enzyme catalytic site are involved in T_4 to T_3 conversion in mammals (Visser, 1979). Pimlott and Eales (1983), Shields and Eales (1986) and this study have shown that exogenous thiol (DTT) is required for significant activity of 5'D. To determine the kinetic mechanism of 5'D, both thiol cofactor and substrate concentrations were varied. For the double-reciprocal plots of liver, gill and muscle 5'D (low substrate enzyme), the various DTT levels were represented by a series of parallel lines, demonstrating that DTT affects the K_m of the enzyme, and that deiodination takes place through a ping-pong,

mechanism, which is a property of mammalian Type I 5'D (Goswami and Rosenberg, 1984). This ping-pong mechanism is characterized by the transiodination and reduction of a sulfenyl-I enzyme intermediate by a thiol cofactor (Visser, 1979; Leonard and Rosenberg, 1980). The present data support the view that a comparable mechanism is occurring at low T_4 concentrations in at least liver, gill and muscle tissues of rainbow trout, and possibly other teleosts.

In liver and kidney at high T_4 concentrations, DTT does not affect the K_m of the enzyme. This parallels the mammalian Type II 5'D in which the thiol, iodothyronine and enzyme form a complex (Visser et al, 1983), representing a sequential mechanism.

Thiouracil analogs can interact with enzyme sulfhydryls to form a mixed disulfide, effectively inhibiting the reaction. This inhibition occurs in the mammalian Type I 5'D system which has a ping-pong mechanism (Chopra et al, 1982). It does not occur in the Type II reaction, and PTU insensitivity reflects a lack of participation of sulfhydryl groups in the mechanism, which is considered to be sequential (Visser et al, 1983). The PTU inhibition demonstrated over the low substrate range for liver, gill and muscle in the present study supports the thiol activation results in suggesting a ping-pong type mechanism. In contrast, the high substrate enzyme was unaffected by PTU in liver and kidney, supporting the suggestion of a sequential, rather than a ping-

pong, mechanism.

In general, these experiments in rainbow trout have identified two types of 5'D in teleost tissues. The high substrate enzyme occurs in kidney and liver, and requires twice as much DTT as the low substrate enzyme. The DTT affects the 5'D K_m, and PTU has no effect. Thus this enzyme resembles the sequential mechanism of the mammalian Type II 5'D. On the other hand, the low substrate enzyme occurs in liver, gill and muscle, and requires less DTT than the high substrate enzyme. Furthermore, DTT does not affect the K_m of this enzyme system, and PTU is uncompetitive with the substrate, indicating a ping-pong mechanism similar to that of the mammalian Type I 5'D. However, the substrate requirements for the two 5'D systems vary from those in mammals. The high Km (Type I) mammalian enzyme operates through a mechanism like that of the low substrate enzyme in rainbow trout, and the low K_m (Type II) mammalian enzyme operates through a mechanism comparable to the high substrate enzyme in rainbow trout. Thus, the enzyme most closely resembling the mammalian Type I enzyme may be the principal deiodinating reaction in fish, as it operates, in trout at least, at substrate levels which are probably in the physiological range.

CHAPTER 2

THE EFFECTS OF SHORT-TERM TREATMENT WITH TESTOSTERONE AND ESTRADIOL ON HEPATIC 5'D ACTIVITY AND PLASMA T_3

INTRODUCTION

Interrelationships between reproductive status and thyroid status in fish have been suggested by several authors. Both androgens and estrogens have been linked to effects on the thyroid system based on both direct studies involving experimental manipulation of reproductive status (Singh, 1969; Sage and Bromage, 1970; Van Overbeeke and McBride, 1971; Singh, 1978; Singh and Raizda, 1979; Hunt and Eales, 1979; Milne and Leatherland, 1980; Olivereau et al, 1981; Chakraborti et al, 1983; Chakraborti and Bhattacharya, 1984; Leatherland, 1985) and seasonal correlations (White and Henderson, 1977; Osborn and Simpson, 1978; Osborn et al, 1978; Leatherland and Sonstegard, 1980; 1981; Eales and Fletcher, 1982; Sower and Schreck, 1982; Burke and Leatherland, 1983; Biddiscombe and Idler, 1983 and Ueda et al, 1984; Cyr et al, 1988a). However, the relationships are poorly understood and, for example, in the rainbow trout, inconsistencies exist between independent studies on the effect of testosterone (T) on the thyroid system. On the one hand, plasma T_3 levels have been shown to increase, along with a possible increase in plasma T_4 upon injection of testosterone proprionate (TP)

(Hunt and Eales, 1979). On the other hand, Milne and Leatherland (1980) showed no effect of methyl testosterone (MT) on plasma thyroid hormone levels, and Leatherland (1985) showed MT to significantly lower plasma T_3 levels with no effect on plasma T_4 levels. 17β-estradiol (E2) has also been shown to either enhance thyroid activity (Singh, 1978; Chakraborti et al, 1983) or depress it (Olivereau et al, 1981; Leatherland, 1985) depending on the species of fish studied.

In light of the apparent inconsistent data available on the effect of E2 and testosterone and its various forms on the thyroid system in salmonids, this study examines the effects of androgen injections (T, MT and TP) on the thyroid system in the Arctic charr (Salvelinus alpinus) and the effects of E2 injections in the rainbow trout. The specific objective was to determine the extent to which short-term treatment of salmonids with androgens or estradiol modifies T_4 deiodination to T_3 as indicated by plasma T_3 levels and/or the activity of hepatic microsomal T_4 5'D.

MATERIALS AND METHODS

Fish Maintenance:

Immature fish were obtained from the Rockwood hatchery (Balmoral Manitoba) and held in tanks with continuously flowing dechlorinated city water at 12°C under a constant photoperiod (12-h L:12-h D), except Trial II fish which were

held under 24-h L. The fish were fed 1-2% of body weight (Trout Grower Pellets, Martin Feed Mills, Ontario) between 0830 and 0900 h. Fish were acclimated for at least one week prior to the start of an experiment.

Experimental Protocol:

Anaesthetized fish (0.1 g/L MS222) were injected on Day 0 and Day 3 with either T (Trials I, II and III), MT (Trials II and III), TP (Trials II and III) or E2 (Trials IV and V) at a dose of 0.5 mg/100 g in 100 µL of peanut oil. (All hormones, Sigma Chemicals, St. Louis.) Controls received 100 µL of peanut oil alone. Fish were injected between 0830 and 0930 h on days 0 and 3 and were sampled between 0830 and 1000 h on days 7 and 12. They were fed a 1% ration during the experimental period and all groups consumed food during this time. Blood samples were removed from the caudal vessels using a heparinized syringe. Plasma was separated by centrifugation and stored at -70°C. After removal of blood samples, the fish were killed by concussion and their livers were removed and rinsed with ice-cold buffer (0.1 M KH_2PO_4/Na_2HPO_4, 0.25 M sucrose, 3 mM DTT, 5mM EDTA, pH 7.2). Weighed livers in each experimental group were combined in Trials I, IV and V. In Trials II and III, sub-samples consisting of half the livers in each experimental group were combined. The microsomal fractions were separated by centrifugation and stored at -70°C (Shields and Eales, 1986).

Testosterone RIA:

Plasma T levels were measured in Trials I and III. Three mL of ethyl acetate:hexane (3:2, v/v) were added to 200 uL of plasma and vortexed 3 times for 20 s at 5-min intervals. One millilitre of solvent was withdrawn from each tube, transferred to another tube and evaporated to dryness. The sample was reconstituted in 1 mL RIA buffer (0.5 M Tris, 0.1 M NaCl, 0.1% gelatin, 0.016 M NaN_3; pH 7.9), vortexed, and let stand for 30 min with occasional vortexing. Two-hundred mL of standard (T, MT, or TP 1.5 to 50 ng/mL) in RIA buffer) or samples were added to microcentrifuge tubes to which 100 uL of ^{125}I-T [Testosterone-6-(0-carboxymethyl)oxima-(2-^{125}I-iodohistamine); 0.007 uCi; sp act 2000 uCi/mmol] dissolved in RIA buffer was added along with 100uL of T-antiserum (Calchemical, Calgary). The binding of the various forms ot testosterone to the T-antiserum did not differ significantly ($p<0.05$). Tubes were capped and incubated overnight at 4oC. Incubation was ended by adding 200 uL dextran coated charcoal (0.625 g Norite A: 0.0625 g dextran in 100 mL of RIA buffer) followed by 20-min incubation at 4oC.

The supernatant was pipetted into a counting tube and the radioactivity was determined. Data were corrected for extraction efficiency which exceeded 97%.

Thyroid Hormone RIA:

Plasma T_3 and T_4 concentrations were determined simultaneously using a modified procedure of Omelanjiuk et al (1984). Five-mL Quik-Sep columns (Isolab, Inc., Ohio) with 0.3 g (dry weight) of G-25 Sephadex were used. The plasma T_4 was determined first (incubation time 3h, barbital buffer, pH 8.6) followed by T_3 (incubation time 16h, phosphate buffer - 26.8 g/L $Na_2HPO_4.7H_2O$; 11.2 g/L Na_2EDTA, pH 7.4; White and Henderson, 1977).

T_4 5'-Monodeiodinase Assay:

5'D was evaluated by $^{125}I^-$ generation following microsome incubation with [^{125}I]T_4. The assay was based on the method of Shields and Eales (1986). Thawed microsomal fractions were diluted 1:19 (v/v) with ice-cold buffer. DTT was added to a concentration of 5 mM (as per Shields and Eales, 1986), and the assay was performed at 12°C, pH 7.2 for an incubation time of 30 minutes. Triplicate tubes were run for each T_4 concentration (10 to 132 nM). Lineweaver-Burk plots for 5'D were calculated by the least squares method. Protein concentrations of the incubates were determined using the Bio-Rad protein assay kit (Bradford, 1976).

Statistics:

Statistically significant differences between mean plasma levels of thyroid hormone in experimental and control groups were determined by analysis of variance (ANOVA) (Mendenhall, 1983).

RESULTS

Androgen treatment:

Plasma testosterone levels were increased ($P<0.025$) over the corresponding control groups in Trial I (Day 7 and 12) and Trial III (Day 7 and all but the TP treatment Day 12) (Table 2).

In all three trials the administration of T or its derivatives increased plasma T_3 levels on Day 7. The stimulation of the plasma T_3 continued through to Day 12 in all but the TP injection of Trial III (Table 3). There was an inconsistent decrease in plasma T_4 on Day 7 which returned to normal on Day 12. Plasma T_4/T_3 ratios (Table 3) were significantly lower than controls in all experimental groups. This effect appeared to be caused primarily by higher T_3 levels in experimental fish, but was influenced by lower T_4 levels in some cases. The HSI (hepatosomatic index; liver weight X 100/ body weight) values were unchanged by the androgen treatments.

Hepatic 5'D was consistently stimulated by androgen in all three trials on days 7 and 12, although the degree of stimulation differed between the forms of testosterone. The Vmax increased in all experimental groups. The extent of the increase ranged from 1.3 times control [Trial II, Day 12, T (ii)] to 5.9 times control [Trial III, Day 7, MT (i)] (Table 4). Changes in Km were small and not always related to the experimental condition.

Table 2. Plasma androgen levels ($\bar{X} \pm$ S.E.M.) in arctic charr injected ip with testosterone (T), testosterone propionate (TP), or 17α-methyl testosterone (MT) (0.5mg/100g) on days 0 and 3. n=8.

Trial	Day	Treatment	Plasma testosterone equivalents (ng/mL)
I	7	C	26 ± 10
		T	282 ± 97*
	12	C	37 ± 33
		T	312 ± 44*
III	7	C	26 ± 10
		T	276 ± 57*
		MT	166 ± 15*
		TP	404 ± 76*
	12	C	27 ± 9
		T	258 ± 77*
		MT	127 ± 37*
		TP	220 ± 97

*$p<0.025$

Table 3. Effects of androgens on HSI, plasma T_3 and plasma T_4 levels and T_4/T_3 ratios in arctic charr. Charr were injected ip on days 0 and 3 with 0.5 mg/100 g of testosterone (T), testosterone propionate (TP) or 17α-methyl testosterone (MT) in 100 L of peanut oil. Controls (C) received peanut oil alone. Data are expressed as $\bar{X} \pm$ S.E.M.

Trial	Day	Treatment	n	Fish Weight (g)	HSI (Liver wt/body wt X 100)	Plasma T_3 (ng/mL)	Plasma T_4 (ng/mL)	Plasma T_4/T_3
I	7	C	15	245±10.2	2.0±0.09	1.75±0.26	1.32±0.04	0.92±0.18
		T	15	270±14.0	2.0±0.07	2.87±0.24*	0.98±0.09*	0.39±0.04*
	12	C	13	232±20.0	1.4±0.14	2.07±0.22	1.53±0.27	0.80±0.15
		T	15	263±15.8	1.3±0.07	3.65±0.31*	1.51±0.20	0.48±0.06
II	7	C	13	216±20.7	1.3±0.12	1.01±0.07	1.23±0.16	1.24±0.18
		MT	11	205±25.8	1.2±0.08	1.55±0.10*	0.58±0.09*	0.53±0.05*
		T	12	214±16.9	1.2±0.08	1.68±0.21*	0.91±0.08*	0.73±0.10*
		TP	12	195±27.9	1.1±0.10	1.56±0.15*	0.90±0.18	0.76±0.13*
	12	C	11	126±14.2	1.3±0.10	1.58±0.48	1.49±0.21	1.46±0.23
		MT	11	148±16.7	1.1±0.05	3.77±0.45*	1.71±0.23	0.51±0.06*
		T	12	124±10.7	1.2±0.05	2.62±0.44*	1.58±0.15	0.69±0.08*
		TP	11	119±10.8	1.2±0.08	2.50±0.40*	1.43±0.17	0.64±0.08*
III	7	C	11	245±13.8	1.5±0.07	1.84±0.50	2.53±0.45	0.98±0.12
		MT	12	234± 6.3	1.7±0.14	4.09±0.50*	1.62±0.68*	0.38±0.02*
		T	10	245±10.3	1.6±0.13	3.27±0.61*	2.04±2.73	0.54±0.06*
		TP	11	237± 8.4	1.7±0.08	2.89±0.35*	0.94±0.24*	0.35±0.07*
	12	C	10	308±12.5	1.8±0.05	2.80±0.38	2.00±0.38	0.74±0.10
		MT	10	329±13.0	1.8±0.10	5.14±0.16*	2.01±0.22	0.36±0.08*
		T	11	328±11.8	1.8±0.05	4.54±0.59*	1.82±0.15	0.50±0.14*
		TP	11	305±14.3	1.8±0.06	2.82±0.27	1.46±0.10*	0.49±0.05*

*$p<0.05$ ANOVA significantly different from controls

Table 4. V_{max} and K_m values for hepatic T_4 5'D of control fish (C) or fish injected (ip) on days 0 and 3 with 0.5mg/100g of testosterone (T), testosterone propionate (TP), or 17α-methyl testosterone (MT).

Trial	Day	Treatment	V_{max}[a] Pool (i)	V_{max}[a] Pool (ii)	K_m[b] Pool (i)	K_m[b] Pool (ii)
I	7	C	4.4	--	1.7	--
		T	8.7	--	2.2	--
	12	C	3.9	--	1.5	--
		T	6.2	--	1.5	--
II	7	C	3.5	3.8	0.86	0.84
		T	5.7	9.6	1.4	1.9
		MT	5.0	5.4	1.3	1.1
		TP	5.1	5.9	1.3	0.83
	12	C	---	3.6	---	1.2
		T	6.1	4.8	1.6	9.2
		MT	7.6	5.6	1.0	1.4
		TP	6.1	6.0	1.2	1.7
III	7	C	2.6	2.4	1.0	1.0
		T	7.2	3.6	0.90	0.92
		MT	15.4	9.0	1.2	2.5
		TP	5.0	4.0	1.1	1.0
	12	C	2.0	2.2	1.5	1.4
		T	6.4	6.0	1.1	1.3
		MT	3.3	4.0	1.0	1.3
		TP	---	4.3	---	1.7

Note: For most treatments two separate liver pools (i) and (ii) were analyzed.
[a]V_{max} represents amount of functional deiodinase [(pmol T_4 conv'd/h/mg prot)].
[b]K_m represents enzyme-substrate affinity [X 10^{-9}M].

Estradiol Treatment:

Hepatic 5'D in E2-treated trout was consistently depressed on Day 7 (Figure 17). This effect was the result of a 10-fold decrease in V_{max} while K_m values were slightly decreased (Table 5). In both trials 5'D returned to control levels by Day 12.

DISCUSSION

Androgen treatment:

The injection protocol used in this study raised plasma T levels from 26-37 ng/mL (controls) to 166-404 ng/mL (androgen-treated). Plasma T was maintained at a high level in the experimental groups (127-312 ng/mL) until Day 12, indicating the effectiveness of the double injection protocol for creating a sustained high T level and also implying a slow T clearance from plasma in these immature charr. The plasma T levels of the androgen-treated charr corresponded to T levels observed in sexually maturing salmonids (Whitehead and Bromage, 1980). The dose of 0.5 mg/100g falls in the general range of that used by Hunt and Eales (1979) and Leatherland (1985) to investigate androgen effects on salmonid thyroid function.

Two injections of T, MT or TP into arctic charr significantly increased plasma T_3. This agrees with the report of Hunt and Eales (1979) in which TP injections (five injections at 4-day intervals) into immature rainbow trout

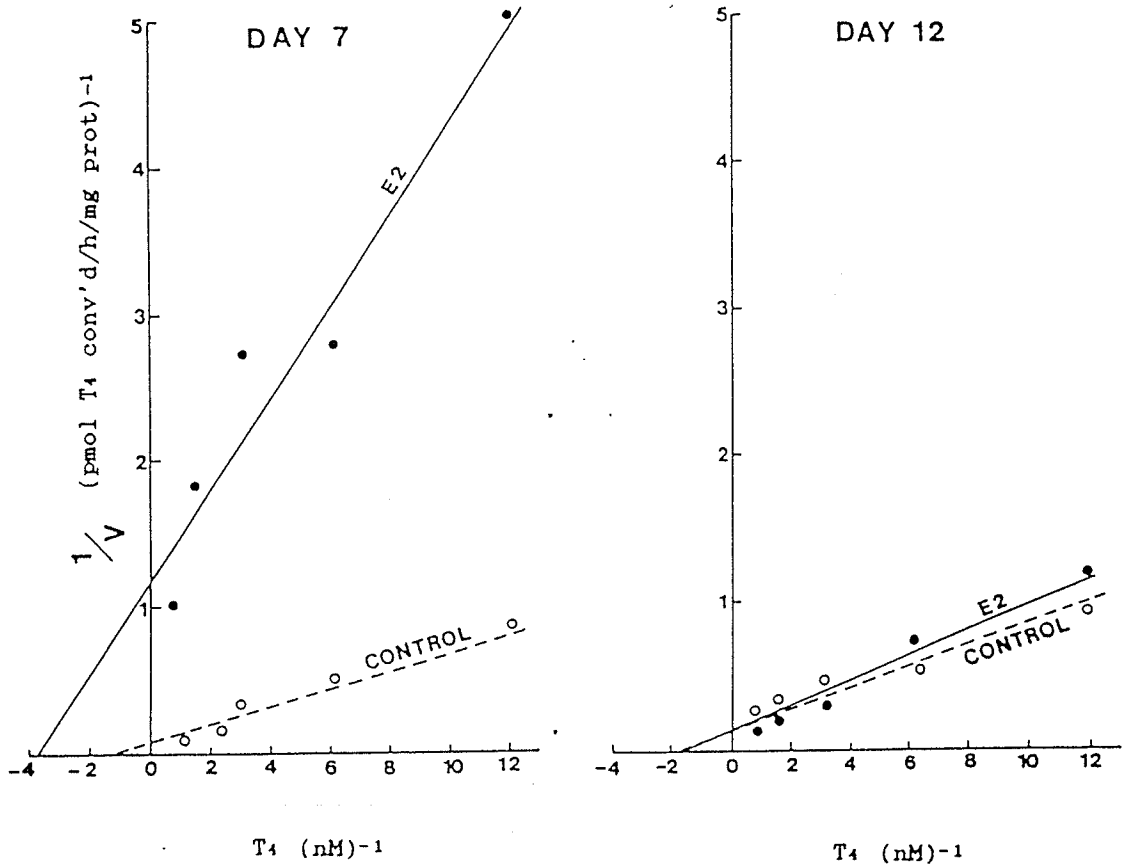
Table 5. V_{max} and K_m values for hepatic T_4 5'D of control fish (C) or fish injected (ip) on days 0 and 3 with 0.5mg/100g 17β-estradiol (E2).

Trial	Day	Treatment	V_{max}[1]	K_m[2]
IV	7	C	7.9	0.77
		E2	0.87	0.49
	12	C	10.7	0.93
		E2	11.4	0.92
V	7	C	9.4	0.70
		E2	0.86	0.26
	12	C	7.0	0.51
		E2	7.9	0.69

[1]pmol T_4 conv'd/ h/ mg prot

[2]10^{-9}M

Figure 17. Lineweaver-Burk plots of hepatic T_4 5'D from trout treated with E2 or peanut-oil controls. Trout were sampled on days 7 and 12. V_{max} and K_m values are given in Table 5.


DAY 7
DAY 12
1/V (pmol T4 conv'd/h/mg prot)-1
E2
CONTROL
T4 (nM)-1
T4 (nM)-1

also caused an increase in plasma T_3, but disagrees with previous studies on MT injection into rainbow trout (Milne and Leatherland, 1980 and Leatherland, 1985). In the latter studies plasma T_3 levels were either lowered or unaltered by MT. Apart from differences in species, time-courses and dosages between the studies, no explanation for this discrepancy exists.

T_4 levels were either unaltered or decreased by androgens in this study. Both Milne and Leatherland (1980) and Leatherland (1985) found no change in T_4 levels due to MT-injection. These results contrast with those of Hunt and Eales (1979) in which TP was usually found to increase plasma T_4. Hunt and Eales (1979), however, also showed that TP stimulated the plasma clearance of T_4, which could account for the decreased T_4 levels seen in this study.

The deiodinase Km varied from 0.9-2.5 X 10^{-9} M with no indication of influence by androgen treatment. This is in contrast to a previous study on rainbow trout in which slight decreases in Km were found to be associated with decreases in Vmax in starved trout (Shields and Eales, 1986).

$_A$n increase in Vmax (amount of functional enzyme) is mainly responsible for the increased deiodinase activity due to androgen treatment. No other vertebrate studies are available for comparison. The form of testosterone did not alter the extent of deiodinase stimulation. It is possible that androgens have stimulated increased synthesis of T_4 5'D.

Present data suggest that thyroidal status is increased by androgens which stimulate both plasma T_3 and T_4 5'D. Enhanced thyroidal status due to androgens agrees with previous findings in rainbow trout (Hunt and Eales, 1979), other teleosts (Singh, 1969; Sage and Bromage, 1970; Van Overbeeeke and McBride, 1971 and Singh and Raizda, 1977) and also rats (Harris et al., 1979) in which T stimulated in vivo T_4 to T_3 conversion.

The present data indicate no consistent differences in potency of T, MT, or TP in elevating plasma T_3 or 5'D. In mammals, the addition of both propionate and methyl groups to testosterone alters the metabolic clearance rate and biological potency (Sellers, 1980). However, at the plasma androgen levels prevailing at 7 and 12 days postinjection, maximal stimulation of 5'D may be occurring in the immature charr used in this study, providing little scope for discrimination of potency between testosterone forms.

The physiological significance of the testosterone effects on the increase in plasma T_3, hepatic T_4 5'D activity, and posssibly thyroid function as a whole may be linked to the anabolic actions of T (Higgs et al, 1977; Fagerlund et al, 1980). Higgs et al (1977), Fagerlund et al (1980), Higgs et al (1982) and McBride et al (1982) have shown MT and either T_4 or T_3 to be synergistic in promoting salmonid growth. The mode of anabolic action of testosterone derivatives may be to increase T_3 levels, as T_3 alone is a growth promotor (Fagerlund

et al, 1980). The role of thyroid hormones in promoting growth has also been suggested to be a permissive one in which thyroid hormones act in conjunction with other anabolic hormones (Eales, 1979). The relationship between T and T_3 may be important in the natural life of a salmonid, as in hatchery-reared rainbow trout seasonal correlations between increasing plasma levels of T and T_3 have been noted (Cyr et al, 1988a).

While T administration stimulates thyroid activity in charr, the present data do not indicate whether T or a metabolite, for example E2 produced from T by aromatization, is the active compound at the cellular level. High levels of E2 inhibit thyroidal status, but the possibility cannot be excluded that low E2 levels generated from T may enhance thyroid function (Singh, 1978; Chakraborti et al, 1983; Chakraborti and Bhattacharya, 1984; Sower et al, 1984; Flett and Leatherland, 1987). Since the HSI was uninfluenced by T it is unlikely that large amounts of E2 were produced by aromatase in the present study.

Estradiol treatment:

While testosterone administration stimulates thyroid activity in charr, E2 depresses plasma T_3 and the activity of the 5'D enzyme in rainbow trout. E2 caused a consistent depression in plasma T_3 (as assayed by Dr. Daniel Cyr; see Cyr et al, 1988b) as well as a marked depression in 5'D activity. Since all T_3 in trout is probably generated extrathyroidally

by 5'D (Eales, 1985), any action of E2 to depress hepatic 5'D would contribute to a lower plasma T_3 level. Since the turnover rate of hepatic deiodinase has been suggested to occur rapidly in fish (DeLuze et al, 1984), E2 may have depressed the de novo synthesis of cellular 5'D. Present data also suggest that turnover of 5'D is rapid, since by Day 12 the 5'D of the trout treated with E2 on days 0 and 3 was identical to that of control trout.

The slight decrease in K_m of 5'D due to E2 treatment suggests that the affinity of the 5'D for T_4 might be enhanced somewhat by E2. A comparable small decrease in K_m and major decrease in V_{max} were also observed in hepatic 5'D of trout due to starvation (Shields and Eales, 1986). There may be a general tendency for the affinity of 5'D to be increased (K_m to decrease) under conditions where 5'D is less abundant.

Changes in plasma T_3 levels could be accounted for by (i) changes in T_3 clearance, (ii) changes in plasma binding proteins or (iii) changes in T_4 conversion to T_3. E2 has been shown to have no major effect on T_3 plasma clearance rate (Cyr and Eales, 1990), but does increase the capacity of high-affinity and low- capacity T_3 plasma binding proteins (Cyr and Eales, 1989). Changes in deiodination rate therefore play an important role in determining the level of plasma T_3. To date, all T_3 generated in salmonids has been shown to be extrathyroidally derived (Chan and Eales, 1975; Brown et al., 1978; Milne and Leatherland, 1978 and Eales, 1985), and the

liver is the site of the greatest activity of deiodinase (activity/mg protein) (Chapter 1). The consistent changes in hepatic 5'D and could therefore play a significant role in the increase or decrease seen in plasma T_3 levels in testosterone or E2-injected fish.

The physiological significance of E2 inhibition of 5'D and depression of plasma T_3 may relate to the specific functions of these hormones. In salmonid fish, somatic growth and gonadal development take place sequentially, rather than simultaneously. This pattern may exist to allow for the partitioning of energy resources between these two anabolic processes. As ovarian maturation proceeds and vitellogenesis begins, the rising E2 levels may suppress T_3 formation and hence suppress somatic growth in favour of vitellogenesis. Thus interactions between thyroid and gonadal hormones may play a role in directing energy partitioning between somatic and gonadal growth.

In conclusion, all three androgens tested stimulated hepatic 5'D activity and the level of plasma T_3. The physiological link between these hormones may be due to their metabolic roles in promoting growth and metabolism. The estrogen E2 acts to decrease 5'D activity by a 10-fold decrease in V_{max} and a slight decrease in K_m. The partitioning of resources in vitellogenic female fish could account for the decrease in conversion of T_4 to T_3 in estradiol-treated fish.

CHAPTER 3

EFFECTS OF GROWTH HORMONE ON 5'D ACTIVITY AND PLASMA T_3 LEVELS IN RAINBOW TROUT

INTRODUCTION

Studies in teleosts have shown growth hormone (GH) to be both thyrotropic (Milne and Leatherland, 1978; Grau and Stetson, 1979) and stimulatory to peripheral conversion of T_4 to T_3 as suggested by its effects on plasma thyroid hormone levels and labelled T_4 kinetics (DeLuze and Leloup, 1982; 1984; DeLuze et al, 1987). In humans (Rezvani et al, 1981; Sherman et al, 1987; Grunfeld et al, 1988), GH increased extrathyroidal conversion of T_4 to T_3 as measured by increased T_3 levels, and in chickens (Kuhn et al, 1987) an increase in peripheral deiodination following GH administration was directly shown by measuring the increased activity of hepatic 5'D.

In teleosts, there is as yet no direct evidence that GH changes the activity of 5'D, and the present study was designed to determine the response of hepatic 5'D to administered GH. Human GH (hGH) was injected into immature rainbow trout for the measurement of dose and time responses of 5'D and plasma T_3 levels to GH. The effects of salmon GH (sGH) on 5'D were also investigated.

MATERIALS AND METHODS

Fish Maintenance:

Rainbow trout were held under the same conditions as in Chapter 2, with the exception that they were fed 2% of body weight per day.

Experimental Protocol:

Trout were anaesthetized in their tanks by pouring in a concentrated solution of MS222 to generate an ambient concentration of 0.1 g/L. Anaesthetized trout were injected intraperitoneally at time 0 (between 0800 and 0900 h) with hGH (Sigma) or sGH (Dr. H. Kawauchi, Laboratory of Molecular Endocrinology, Kitasato University, Japan) in 20 µL CAPS buffer, pH 9.1. Controls received 20 uL of CAPS buffer alone. Depending on the experiment, trout were sampled at 5 to 48 h post-injection (pi). Anaesthetized fish were weighed and blood was removed from the caudal vessels using a heparinized syringe. Plasma was separated by centrifugation and stored at -70°C. After bleeding, trout were killed by concussion and their livers were removed, weighed, and rinsed with ice-cold buffer (0.1 M KH_2PO_4/Na_2HPO_4, 0.25 M sucrose, 3 mM DTT, 5 mM EDTA; pH 7.2). Within any given experimental group, weighed livers were combined into two pools. The microsomal fractions were separated by centrifugation and stored at -70°C (Shields and Eales, 1986). The hepatosomatic index was calculated for each fish.

Thyroid hormone RIA:

This was performed as in Chapter 2.

5'-Monodeiodinase Assay:

This was performed as in Chapter 2.

Statistics:

Statistically significant differences between mean plasma levels of hormones or enzyme activities for experimental and control groups for Experiments 1-3 were determined by analysis of variance (ANOVA). For Experiments 4 and 5, the Student-Neuman-Keuls' (SNK) test was used to determine significant differences between the means of all the treatment groups (Steel and Torrie, 1980).

RESULTS

Experiment 1:

Trout were injected with 0.4 µg hGH/g and sampled together with controls at 24 and 48 h pi. At both times plasma T_3 and Vmax were increased significantly by hGH with no change in T_4, Km or HSI (Table 6).

Experiment 2:

Trout were injected with 0.4 µg hGH/g and sampled together with controls at 6, 12, 24, and 48 h pi. Except at 6 h pi, no significant changes occurred in T_4 with hGH injection (Figure 18). Km (controls, 1.14-1.25 X 10^{-9}M; hGH, 1.14-1.33 X 10^{-9}M) and HSI (controls, 1.41-1.67%; hGH, 1.53-

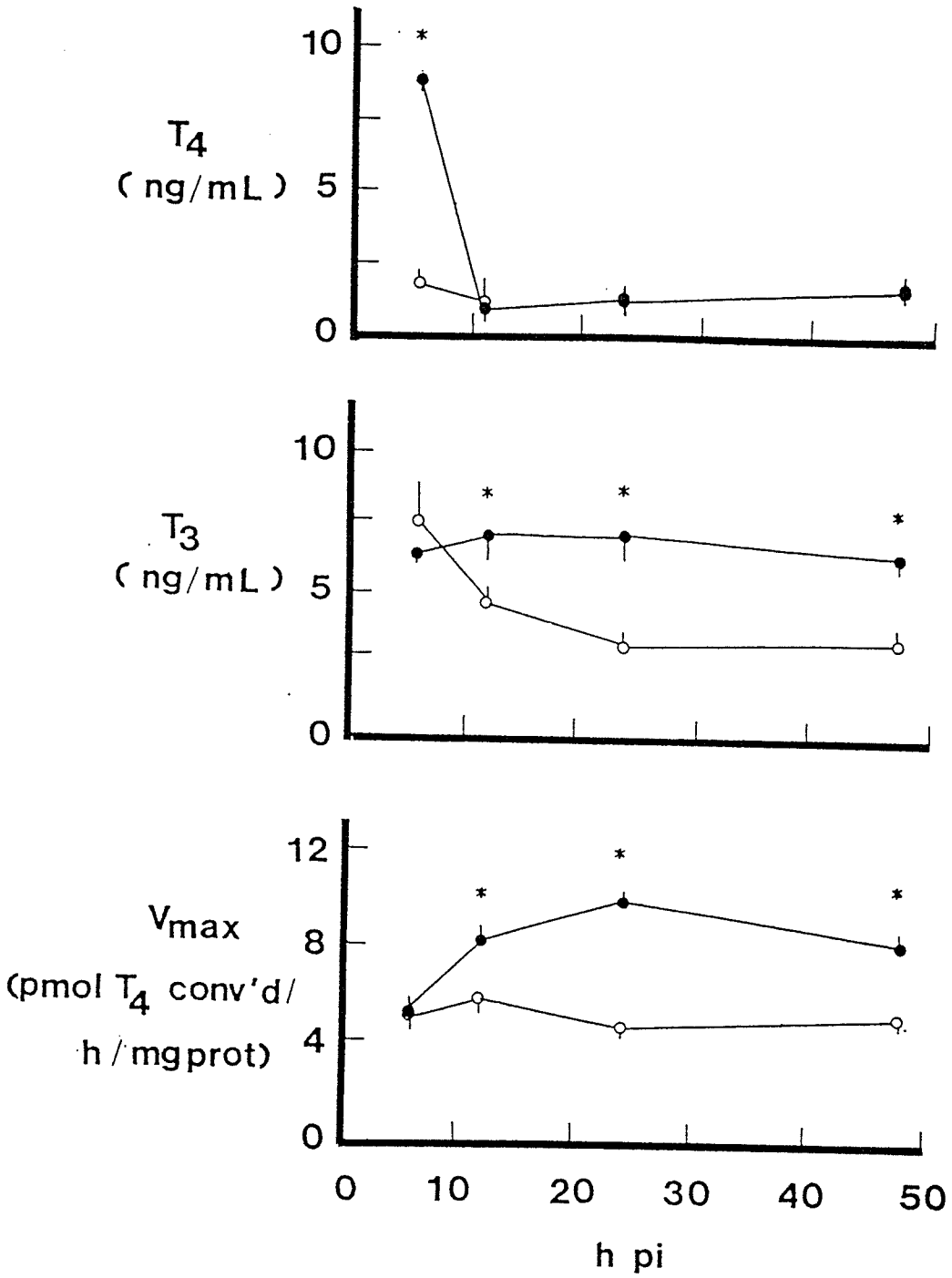
Table 6. Plasma T_4 and T_3 levels and hepatic V_{max} and K_m of rainbow trout sampled 24 or 48 h after injection with CAPS buffer containing human growth hormone (hGH; 0.4 μg/g = E)or buffer alone (controls = C). Values are $\bar{X} \pm$ S.E.M.

		24 h		48 h	
Parameter	n	C	E	C	E
Body weight (g)	15	201.9 ± 12.5	176.5 ± 10.5	204.3 ± 8.7	198.2 ± 13.1
HSI (liver wt x 100/ body wt.)	15	1.12 ± 0.07	1.27 ± 0.08	1.25 ± 0.04	1.38 ± 0.10
Plasma T_4 (ng/mL)	15	4.2 ± 0.69	5.8 ± 1.2	4.5 ± 0.53	4.1 ± 0.62
Plasma T_3 (ng/mL)	15	6.5 ± 0.55	9.5 ± 0.64**	7.0 ± 0.16	8.6 ± 0.56*
V_{max} (pmol T_4 conv'd/h/ mg prot)	2	1.50 ± 0.14	5.03 ± 0.81*	1.85 ± 0.01	4.42 ± 0.55*
K_m ($X\ 10^{-9}$M)	2	1.24 ± 0.03	1.34 ± 0.06	1.35 ± 0.02	1.12 ± 0.05

*p<0.05 significantly different from controls

**p<0.01 significantly different from controls

Figure 18. Plasma T_4 and T_3 levels and V_{max} of hepatic 5'D of rainbow trout injected with human growth hormone (hGH; 0.4 g/g) in CAPS buffer (●) or buffer alone (controls;○) and sampled at 6, 12, 24 or 48 h after injection. Values are the mean ± the S.E.M. n = 12 for T_4 and T_3. n = 2 for V_{max}.


10
5
0
T_4
(ng/mL)
*
10
5
0
T_3
(ng/mL)
*
*
*
12
8
4
0
V_{max}
(pmol T_4 conv'd/
h / mg prot)
*
*
*
0
10
20
30
40
50
h pi

1.60%) showed no significant differences due to hGH. However, significant elevations occurred in T_3 and Vmax of hGH-injected fish at 12, 24 and 48 h pi. Peak values were measured at 24 h pi (Figure 18).

Experiment 3:

Trout were injected with 0.4 µg hGH/g and sampled at 5, 8, 11 and 14 h pi. No significant changes occurred in T_4 (Figure 19), Km (controls, 1.30-1.38 X 10^{-9}M; hGH, 1.14-1.53 X 10^{-9}M) or HSI (controls, 1.26-1.31%; hGH, 1.14-1.34%). Plasma T_3 and Vmax increased significantly in hGh-injected fish at 8, 11 and 14 h pi (Figure 19).

Experiment 4:

Trout were injected with either 0, 0.1, 0.4 or 1.0 µg hGH/g and sampled at 24 h pi. No changes occurred in plasma T_4 levels, HSI or Km. Plasma T_3 levels in fish injected with 0.4 and 1.0 µg hGH/g were significantly different from those for all other groups (Table 7).

Experiment 5:

The protocol of Experiment 4 was followed except that the Vmax and Km of hepatic 5'D were also measured. No significant differences in T_4, Km or HSI were observed between the treatments (Table 8). Plasma T_3 levels did not differ significantly between the 0.4- and 1.0-µg/g doses (0<0.1<0.4=1.0 µg/g). Vmax levels, however, were significantly different between all doses (0<0.1<0.4<1.0 µg/g).

Figure 19. Plasma T_4 and T_3 levels and V_{max} of hepatic 5'D of rainbow trout injected with human growth hormone (hGH; 0.4 g/g) in CAPS buffer (●) or buffer alone (controls;○) and sampled at 5 , 8, 11 or 15 h after injection. Values are the mean ± the S.E.M. n = 10 for T_4 and T_3. n = 2 for V_{max}.

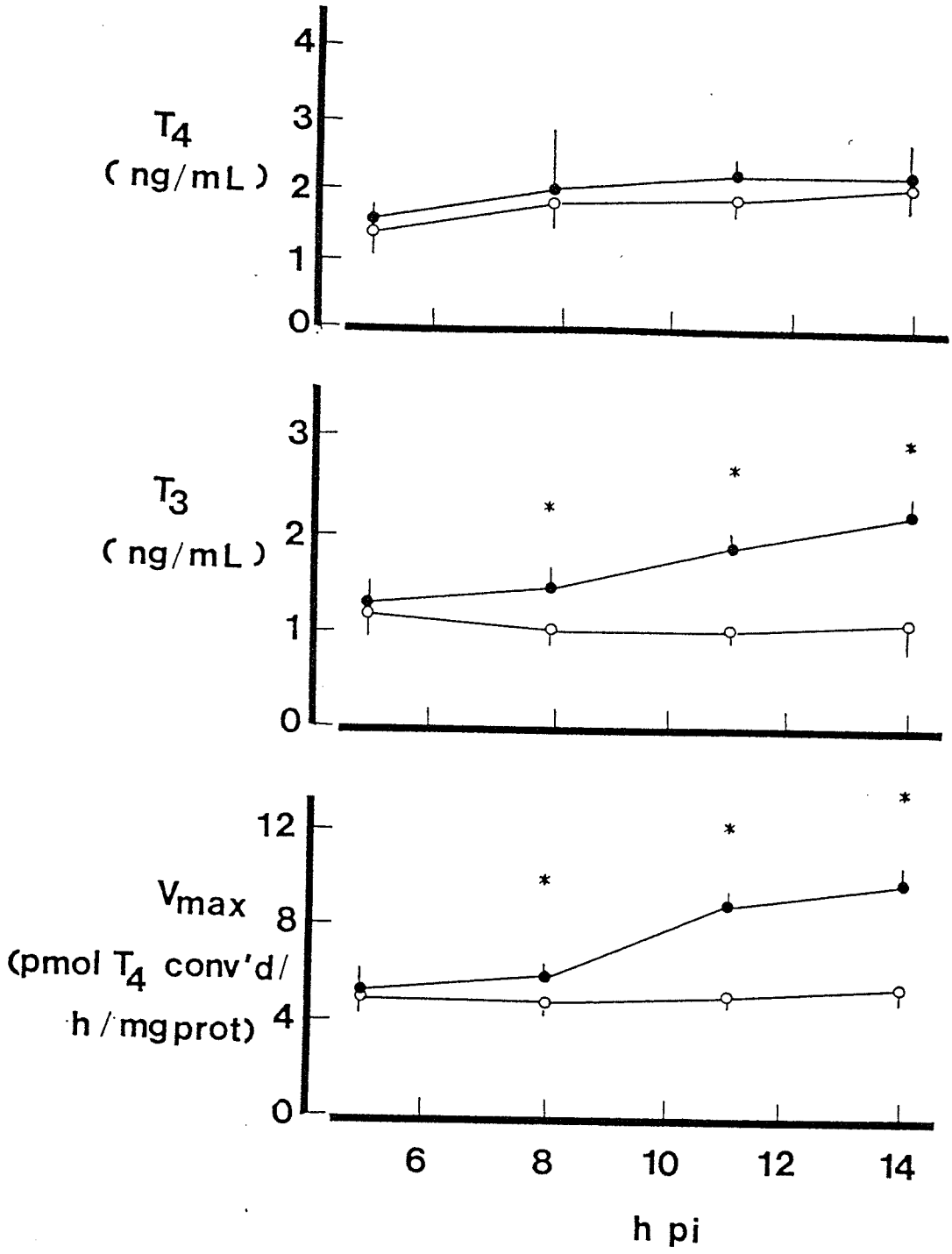

T_4
(ng/mL)
4
3
2
1
0
T_3
(ng/mL)
3
2
1
0
*
*
*
Vmax
(pmol T_4 conv'd/
h /mg prot)
12
8
4
0
*
*
*
6
8
10
12
14
h pi

Table 7. The effects of different doses of human growth hormone (hGH) on plasma T_4 and T_3 levels of rainbow trout measured 24 h after injection (ip). Values are $\bar{X} \pm$ S.E.M. Means with different subscripts differ significantly ($p<0.05$).

Parameter	hGH Dose ($\mu g/g$) 0	0.1	0.4	1.0
Body weight (g)	275.4 ± 17.4	292.7 ± 20.4	273.5 ± 57.9	259.3 ± 20.6
Plasma T_4 (ng/mL)	2.0 ± 0.31[a]	2.4 ± 0.42[a]	2.3 ± 0.61[a]	2.6 ± 0.53[a]
Plasma T_3 (ng/mL)	3.1 ± 0.25[a]	3.5 ± 0.52[a]	5.0 ± 0.28[b]	6.9 ± 0.62[c]

Table 8. The effects of different doses of human growth hormone (hGH) on plasma T_4 and T_3 levels and V_{max} and K_m of hepatic T_4 5'D of rainbow trout measured 24 h after injection (ip). n = 10 for all parameters except V_{max} and K_m where n = 2. Values are X ± S.E.M. Means with different subscripts differ significantly ($p<0.05$).

Parameter	hGH Dose (μg/g) 0	0.1	0.4	1.0
Body weight (g)	198.4 ± 17.2	167.4 ± 17.2	187.8 ± 17.1	195.4 ± 18.1
HSI (liver wt X100/body wt)	1.42 ± 0.20[a]	1.43 ± 0.14[a]	1.52 ± 0.20[a]	1.48 ± 0.20[a]
Plasma T_4 (ng/mL)	1.8 ± 0.46[a]	2.2 ± 0.28[a]	3.4 ± 0.76[a]	1.6 ± 0.53[a]
Plasma T_3 (ng/mL)	3.2 ± 0.37[a]	4.8 ± 0.51[b]	6.3 ± 0.77[c]	6.4 ± 0.45[c]
V_{max} (pmol T_4 conv'd/h/mg prot)	5.20 ± 0.42[a]	7.24 ± 0.48[b]	9.72 ± 0.78[c]	12.7 ± 0.32[1d]
K_m (X 10^{-9} M)	1.31 ± 0.09[a]	1.50 ± 0.11[a]	1.49 ± 0.15[a]	1.28 ± 0.08[a]

Experiment 6:

Trout were injected with sGH at a dose of 0.5 µg/g body weight and sampled 24 h later. Plasma T_4 was not modified but a significant increase in plasma T_3 and a 129% increase in 5'D V_{max} were seen with no change in 5'D K_m (Table 9, Figure 20). HSI did not differ between treatments.

Experiment 7:

Trout injected with sGH (0.1 µg/g body weight) were sampled 24 h later. Plasma T_4 was not modified, but there was a significant increase in plasma T_3 and a 116% in 5'D V_{max}, with no change in 5'D K_m (Table 9, Figure 20). HSI did not differ between treatments.

DISCUSSION

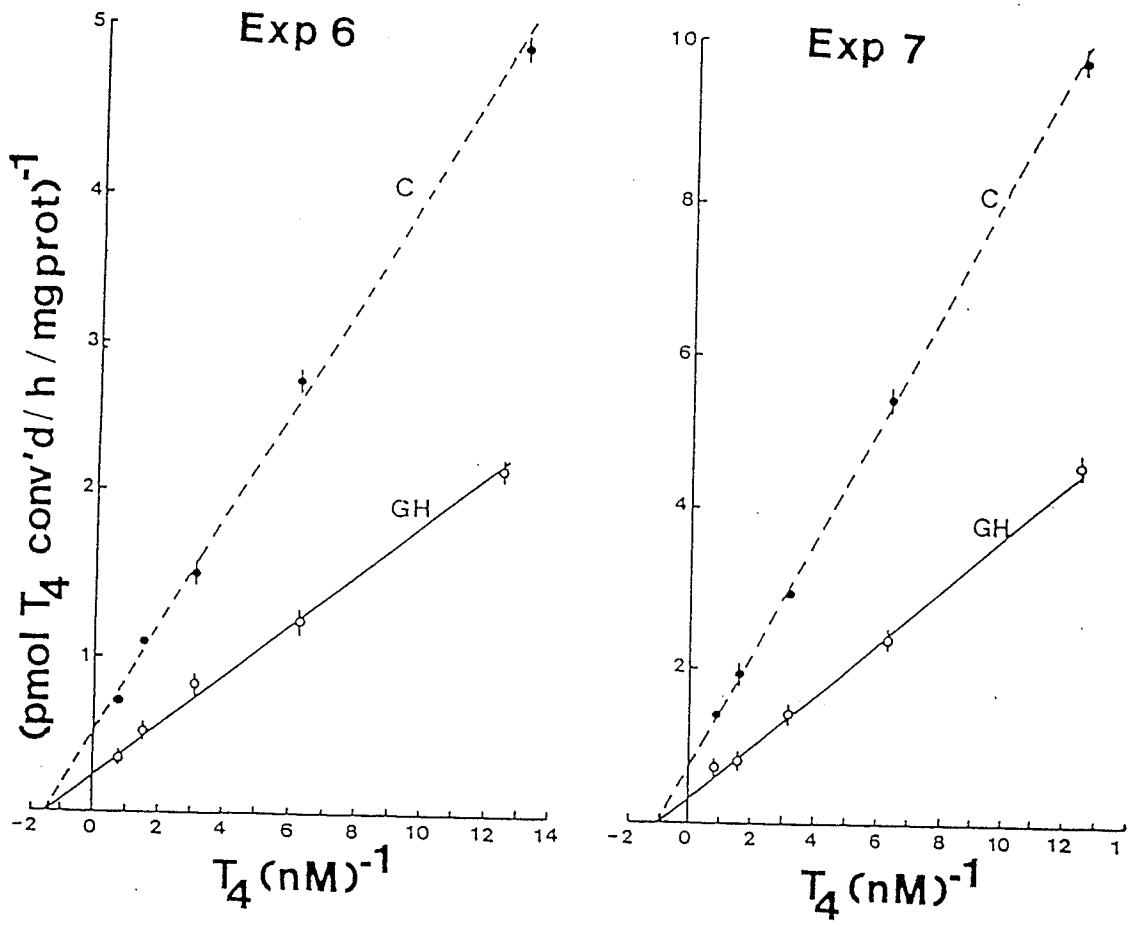
Injections of hGH and sGH (0.1-1.0 µg/g) increased both plasma T_3 levels and the activity (Vmax) of the hepatic 5'D of trout. The increase in Vmax (in hGH-injected fish) was maximal at 24 h pi and was detected significantly as early as 8 h pi. This is the first demonstration of GH stimulation of 5'D Vmax levels. It supports previous *in vivo* studies in the European eel in which both ovine and tilapia GH stimulated T_4 to T_3 conversion, as indicated by the increase in labelled plasma T_3 following injection of labelled T_4 (DeLuze and Leloup, 1982; 1984; DeLuze *et al*, 1987). The present data agree also with those in chickens (Kuhn *et al*, 1987; 1988), where ovine GH and hypothalamic growth hormone-releasing

Table 9. Thyroid parameters for control (C) and experimental (E) trout injected (ip) 24 h previously with buffer or Pacific salmon GH (sGH). n = 10 for all paramaters except V_{max} and K_m where n = 2. Values are means ± S.E.M.

Experiment	sGH dose (μg/g)	Treat-ment	Weight (g)	HSI (%)	Plasma T_4 (ng/mL)	Plasma T_3 (ng/mL)	V_{max} (pmol conv'd/ h/mg prot)	K_m (10^{-9}M)
6	0.5	C	147.6 ± 5.19	1.07 ± 0.10	1.23 ± 0.15	1.03 ± 0.07	1.92 ± 0.04	0.65 ± 0.0045
		E	154.5 ± 5.41	1.10 ± 0.099	0.99 ± 0.08	1.51 ± 0.12*	4.39 ± 0.66*	0.64 ± 0.054
7	0.1	C	123.8 ± 11.8	1.19 ± 0.067	0.92 ± .18	1.48 ± 0.11	1.48 ± 0.17	1.09 ± 0.006
		E	119.3 ± 11.8	1.17 ± 0.092	0.91 ± 0.09	1.93 ± 0.16*	3.19 ± 0.08*	1.04 ± 0.013

*$p<0.05$

Figure 20. Lineweaver-Burk plots of hepatic microsomal 5'D from trout intraperitoneally injected with sGH in CAPS buffer (0.5 μg/g, Experiment 6; 0.1 μg/g, Experiment 7) or buffer alone (C = controls). Trout were killed 24 h after injection. K_m and V_{max} values are given in Table 9. Each point represents the mean $\pm$ S.E.M. of two replicates.


Exp 6
Exp 7
C
GH
C
GH
(pmol T4 conv'd/ h / mg prot)-1
T4 (nM)-1
T4 (nM)-1

factors both caused an increase in hepatic 5'D activity.

The doses of hGH (0.1-1.0 µg/g) used in this study caused graded elevations in both plasma T_3 and 5'D activity. These doses fall within the range (0.06-10 µg/g) used by other reseachers to study GH effects on either the thyroid system or the promotion of growth in teleosts (Markert et al, 1977; Milne and Leatherland, 1978; DeLuze et al, 1987). Based on the responses observed in Experiments 1-3 of this study, a hGH dose of 0.4 µg/g may be considered in the low physiological range. A maximal response to hGH was not determined.

Although the structures of hGH and trout GH are different, it is reasonable that the effects of hGH reflect those of trout GH. Growth hormones isolated from various mammalian and nonmammalian species share many characteristics (Papkoff, 1982). GH from pituitary extracts of teleosts, elasmobranchs and mammals stimulate teleost growth (Donaldson et al, 1979). Numerous studies have shown mammalian GHs promote growth in teleosts (Pickford, 1959; Higgs et al, 1975; Komourdjian et al, 1976; Kayes, 1977; Markert et al, 1977; Weatherley and Gill, 1982). Ovine GH has been used in chickens (Kuhn et al, 1987) and in the eel (DeLuze and Leloup, 1982) to stimulate the peripheral conversion of T_4 to T_3. The effects of mammalian GH on T_4 deiodination in the eel were comparable to those due to tilapia GH (DeLuze et al, 1987). However, the sGH preparation used in the present study appears more potent than the hGh preparation in stimulating both plasma T_3 and hepatic 5'D. The sGH preparation at a dose of

0.1 ug/g caused a significant 116% increase in V_{max}. The same dose of hGH tested under comparable conditions caused a less impressive 39% increase in V_{max}. Furthermore, the sGH doses of 0.1 and 0.5 µg/g (129% increase) were virtually equipotent in stimulating V_{max} , implying action at the the upper plateau of the dose-response curve. In contrast, the hGH preparation progressively increased in effectiveness through the dose-range tested.

The measurements of HSI showed no significant differences in any of the trials between control and experimental groups. Due to the short duration of the experiments (fish sampled 48 h pi or earlier), it is unlikely that any effect that GH has on the liver would result in a weight change. Farbridge and Leatherland (1988) have shown that there is no effect on rainbow trout HSI by ovine GH after a month of administration.

T_4 levels were also unaffected by hGH or sGH injection except in Experiment 2, at 6 h pi. This supports the study of Kuhn et al (1988) in which human pancreatic growth hormone-releasing factor stimulated the release of GH, but was not followed by an increase in T_4. In contrast GH can be thyrotropic in Fundulus heteroclitus (Grau and Stetson, 1979) and rainbow trout (Milne and Leatherland, 1978). However, in those studies, higher doses over a longer period of time were administered than in the present study. Since hGH stimulated T_4 to T_3 conversion, the removal of T_4 from the plasma pool may

be offset by its addition, creating the impression of lack of response of the thyroid to hGH. In support of this, DeLuze and Leloup (1984) have shown that GH in the eel increases the T_4 plasma clearance rate.

Plasma T_4 in rainbow trout may be elevated acutely in response to physically stressful conditions (Brown et al, 1978). While physical disturbance was kept to a minimum in our study, its potential effects have to be taken into account. It is possible that the single instance of T_4 elevation in response to hGH (Experiment 2, 6 h pi) can be explained by inadvertent stress of that particular group, as no differences in plasma T_4 between controls and experimentals in Experiment 3, at 5 and 8 h pi were measured. Far less likely, but impossible to rule out entirely, is the possibility that most of the fish used in the study were physically disturbed to some degree and had greater than normal levels of plasma T_4 which masked any stimulation of plasma T_4 by hGH.

In all 7 experiments, as the level of activity of the enzyme increased, so did the plasma T_3 concentration. Increases in Vmax are responsible for the increased T_4 5'D activity measured in the hGH treated fish. These parallels between hepatic 5'D activity and plasma T_3 imply a causal relationship between hepatic 5'D activity and plasma T_3. This is supported by the study of Cyr et al (1988b), in which the activity of 5'D and plasma T_3 levels were closely correlated

following E2 injection. It is impossible under these experimental conditions to tell whether the decrease in enzyme activity between 24 and 48 h pi is due to a decrease in effect of the hGH or whether the increased T_3 levels are acting to depress the activity of the 5'D enzyme. Autoregulation of T_3 generation by T_3 has recently been shown in fish (following chapter).

The Km of 5'D did not change in response to GH. This is consistent with previous studies in our laboratory, in which Km values did not change markedly following in vivo administration of testosterone (MacLatchy et al, 1988) or E2 (Cyr et al, 1988b). In those studies, any changes in 5'D activity were also accounted for by changes in Vmax. The range in control values of V_{max} indicates, however, that the amount of enzyme present initially in the experiments varied, perhaps due to seasonal differences.

In Experiments 4 and 5, different doses of hGH (0.1, 0.4 and 1.0 µg/g) were administered. Both plasma T_3 and 5'D activity generally increased with increasing hGH dose, although in Experiment 4 the plasma T_3 at 0.1 µg/g was not significantly different from the control values, and in Experiment 5 the 1.0 µg/g dose had no significant effect different from the 0.4 µg/g dose on plasma T_3. These experiments, however, do demonstrate that hGH is able to stimulate 5'D and plasma T_3 levels to a graded degree. This graded response is opposite to the all-or-none response of

tilapia and sockeye salmon (Oncorhynchus nerka) to GH as demonstrated by Clarke et al (1977). The results from the sGH experiments, in which there was little difference between the two doses, demonstrate that sGH and hGH may be somewhat different in their effects, and that sGH may operate using an all-or-none response as previously shown in teleosts.

It is impossible to tell from these experiments whether the effect of GH on the 5'D enzyme is direct or whether it is due to a secondary factor released by GH, such as somatomedin, which is responsible for many of the actions attributed to GH. Somatomedins are produced in Baltic salmon (Salmo salar) (Lindahl et al, 1985), and could be involved in the 5'D response in trout.

Physiologically, the interaction between GH and hepatic 5'D activity and plasma T_3 could be important during growth, as both GH and T_3 have been identified as hormonal stimulants in this process (Higgs et al, 1975; 1977; Markert et al, 1977; Higgs et al, 1979; Weatherley and Gill, 1982; Saunders et al, 1985). In combination, GH and T_4 (Higgs et al, 1977) are more effective in promoting growth than either GH or T_4 alone. Since in trout T_3 is the presumed active thyroid hormone (Eales, 1985), one of GH's important indirect actions in promoting growth in normal fish could be the stimulation of 5'D, which leads to an increase in circulating T_3. Farbridge and Leatherland (1988) have recently demonstrated that ovine GH and T_3 interact in rainbow trout to regulate growth-related

parameters such as condition factor, carcass water content, carcass and liver total lipid content, liver RNA content, plasma fatty acid and plasma protein content.

In conclusion, hGH and sGH have been demonstrated to cause short-term stimulation of T_4 5'D Vmax, resulting in a closely correlated elevation of plasma T_3, with no apparent affect on T_4. The effect of GH on peripheral deiodination could be important in the synergism of GH and thyroid hormones in promoting growth of salmonids. GH can also now be added to the list of hormones which include testosterone (MacLatchy and Eales, 1988; Chapter 2), E2 (Cyr et al, 1988b; Chapter 2) and cortisol (Vijayan et al, 1988) which in salmonids have an affect on the peripheral conversion of T_4 to T_3 at the level of the deiodinase system.

CHAPTER 4

INFLUENCE OF DIETARY LIPID, CARBOHYDRATE, PROTEIN AND T_3 ON 5'D IN RAINBOW TROUT

INTRODUCTION

Nutritional state has been shown to consistently influence T_3 parameters in teleost fish. Starvation for 3 days or longer depresses plasma T_3 levels (Leatherland, 1982; Flood and Eales, 1983; Cook and Eales, 1987), the *in vivo* conversion of injected [^{125}I]T_4 to [^{125}I]T_3 and $^{125}I^-$ (Higgs and Eales, 1977; Eales, 1979) and the V_{max} of the hepatic microsomal 5'D (Shields and Eales, 1986). There is also a decrease in the number of putative T_3 nuclear receptor sites following 3 days or longer of starvation (Van der Kraak and Eales, 1980; Bres *et al*, 1990), further confirming the depressed thyroidal state following caloric deprivation.

In brook trout and arctic charr, correlations between food ration, somatic growth and thyroid function have been established, in which larger rations increase plasma T_3 levels (Higgs and Eales, 1978; 1979; Eales and Shostak, 1985), and studies on rainbow trout have examined the effect of diet on the thyroid and serum thyroid hormones (Leatherland *et al*, 1977; 1980a; 1980b; 1984).

Although those studies have all indicated that nutrient intake modifies the thyroid hormone status of salmonids, there are few studies aimed at identifying the nutritional factors

involved. It has been shown by studying the in vivo kinetics of labelled T_4 that the stimulus for enhanced T_3 generation may be the caloric/protein intake (Higgs and Eales, 1978; 1979). It is also known that alteration of the lipid/carbohydrate (CHO) ratio in isocaloric and isonitrogenous diets does not alter plasma T_3 (Hilton et al, 1988). There has, however, been no evaluation of the effects of diet constituents on 5'D activity.

In this study, three experiments were conducted to investigate, using isocaloric diets, the effects of lipid, CHO and protein on hepatic 5'D activity in rainbow trout. In the first experiment, varying levels of T_3 were also added to the fish food, and the influence of an exogenous T_3 source on hepatic 5'D was evaluated.

MATERIALS AND METHODS

These experiments were conducted at the Rockwood Experimental Fish Hatchery located north of Winnipeg, Manitoba.

Fish Maintenance:

All fish were held in fiberglass tanks (2 X 2 X 2 ft), which received running well water (6 L/min) at 6.5 $\pm$ 0.5°C, and were held under a constant photoperiod (12-h L:12-hD). Trout were fed for one week on their regular diet (Martin Feed Mills, Trout Feed, 1/8") at a ration of 1.5% of wet body weight/day, and then two groups assigned at random to one of

12 diets (Experiment 1) or 6 diets (Experiment 2 and 3).

Experiment 1:

Twenty-four groups of 53 rainbow trout ranging from 24-57g ($\bar{X}$ = 36g) were assigned randomly to tanks in February 1987. The diets were comprised of the three low lipid (LL), medium lipid (ML) and high lipid (HL) diets, each of which contained a supplement of 0, 4, 8, or 12 ppm T_3 (Higgs et al, 1979). The diets were isonitrogenous and isocaloric. The proximate diet compositions are given in Table 10. Diets were fed at satiation from 0900-1600 h for each individual tank. The diets were prepared by D. Higgs (Department of Fisheries and Oceans, Vancouver, B.C.).

In early June, after receiving the experimental diets for 13 weeks, 12 fish from each tank were killed, and their livers were removed and separated into two pools for estimation of hepatic microsomal 5'D activity (see Chapter 2 for 5'D assay method).

Experiment 2:

In May of 1988, twelve groups of fish ($\bar{X}$ = 45.7 g) were assigned randomly to tanks. Six different isocaloric and isonitrogenous diets containing varying levels of protein and lipid, and constant CHO levels were fed in duplicate (Table 11). Four isocaloric diets were fed at 0.97% of body weight and contained protein concentrations at 23, 30, 37 and 44%. The 44% diet was also fed to fish at 0.68% of body weight to

provide a reduced caloric content, but a protein content equivalent to the 30% protein diet. The 44% diet was also fed at 52% of body weight (reduced calories, protein = 23%). The diets were prepared by D. Higgs. Ten fish in each group were sampled following Week 5 of the experiment, their livers removed and separated into two pools which were used for estimation of hepatic microsomal 5'D activity.

Experiment 3:

In July 1988, twelve groups of rainbow trout ($\bar{X}$ = 47.2 g) were assigned randomly to tanks. Six different isocaloric diets (in duplicate) containing varying levels of protein and CHO, and constant lipid levels were fed (Table 12). Four diets contained 45, 30, 15 and 0% protein were fed at 1% of body weight. The 45% protein diet was also fed to fish at 0.67% of body weight, to provide a reduced caloric intake but a protein content equivalent to the 30% protein diet. In addition, the 45% diet was fed at 33% of body weight (reduced calories, protein = 15%). The diets were prepared by D. Higgs. Ten fish were sampled from each group at Week 5 of the experiment, their livers separated into two pools for determination of hepatic 5'D activity.

Statistics:

Differences between levels of a variable were determined by ANOVA followed by the SNK test to determine the differences between individual treatment means (Steel and Torrie, 1980).

Table 10. Composition of diets fed to rainbow trout in Experiment 1. Protein levels are constant, and lipid and carbohydrate levels vary.

Ingredients (g/kg dry weight)	LL	ML	HL
Herring meal[1]	372.5	372.5	372.5
Euphausids (freeze-dried)	50.0	50.0	50.0
Soybean meal	150.0	150.0	150.0
Wheat midlings	70.0	70.0	70.0
α-cellulose	---	74.0	158.2
Cerelose (D-glucose)	268.2	134.2	---
Salmon oil (stabilized)[2]	17.3	77.3	137.3
Vitamin supplement[3]	30.0	30.0	30.0
Mineral supplement[4]	20.0	20.0	20.0
Permapell	15.0	15.0	15.0
Choline chloride (60%)	5.0	5.0	5.0
Ascorbic acid	2.0	2.0	2.0
Estimated level (%) of:			
Protein	39.0	39.0	39.0
Lipid	7.0	13.0	19.0
Carbohydrate	28.3	14.9	1.48
Digestible energy (kcal/kg)[5]	3905	3908	3905
Metabolizable energy (kcal/kg)[6]	3425	3426	3426

[1]Steam-dried whole herring meal.
[2]Stabilized by adding 0.5g santoquin/kg oil.
[3]The vitamin supplement supplied the following levels of nutrients/kg dry diet: Vitamin A acetate, 10 000 IU; cholecalciferol (D_3), 2400 IU DL-alpha-tocopheryl acetate (E), 600 IU; menadione, 26 mg; D-calcium pantothenate, 193 mg; pyridoxine HCl, 44.9 mg; riboflavin, 60 mg; niacin, 300 mg; folic acid, 20 mg; thiamine mononitrate, 40.7 mg; biotin, 3 mg; cyanocobalamin (B_{12}), 0.060 mg; inositol, 400 mg.
[4]The mineral supplement supplied the following levels of minerals/kg dry weight: M_n (as $M_nSO_4.H_2O$), 75 mg; Z_n (as $Z_nSO_4.H_2O$), 50 mg; Co (as $CoCl_2.6H_2O$), 1 mg; Cu (as $CuSO_4.5H_2O$), 5 mg; Fe (as $FeSO_4.7H_2O$), 75 mg; I (as KIO_3), 5 mg; F (as NaF), 4.5 mg; Se (as Na_2SeO_3), 0.11 mg Na (as NaCl), 2063 mg.
[5]Calculated on the basis of the sum of the individual ingredient digestible energy values (Cho et al, 1982).
[6]Calculated according to Beamish et al (1986).

Table 11. Composition of diets fed to rainbow trout in Experiment 2. Protein and lipid levels vary between diets and carbohydrate levels are constant.

Ingredients (g/kg dry weight)	Diet (% Protein) 23	30	37	44
Herring meal	223.0	290.8	358.7	426.5
Euphausids (freeze-dried)	31.8	41.5	51.2	60.8
Soybean meal	104.0	135.7	167.3	199.0
Wheat midlings	33.4	43.6	53.7	63.9
α-cellulose	167.9	112.0	56.0	---
Cerelose (D-glucose)	151.1	148.3	145.6	142.9
Herring oil (stabilized)[1]	156.5	107.7	58.9	10.2
Vitamin supplement[2]	30.0	30.0	30.0	30.0
Mineral supplement[3]	43.9	43.9	43.9	43.9
Permapell	15.0	15.0	15.0	15.0
Choline chloride (60%)	5.0	5.0	5.0	5.0
Ascorbic acid	2.0	2.0	2.0	2.0
DL-methionine	0.43	0.56	0.70	0.83
Calcium phosphate dibasic	36.0	24.0	12.0	---
Estimated carbohydrate (% dry matter)	15.2	15.2	15.2	15.2
Estimated metabolizable energy[4] (kcal/kg)	3300	3300	3300	3300

[1]Stabilized by adding 0.5g ethoxyquin/kg oil.
[2]The vitamin supplement supplied the following levels of nutrients/kg dry diet: Vitamin A acetate, 10 000 IU; cholecalciferol (D_3), 2400 IU DL-alpha-tocopheryl acetate (E), 600 IU; menadione, 26 mg; D-calcium pantothenate, 193 mg; pyridoxine HCl, 44.9 mg; riboflavin, 60 mg; niacin, 300 mg; folic acid, 20 mg; thiamine mononitrate, 40.6 mg; biotin, 3 mg; cyanocobalamin (B_{12}), 0.06 mg; inositol, 400 mg.
[3]See Appendix 3.
[4]Calculated according to Beamish et al (1986).

Table 12. Composition of diets fed to rainbow trout in Experiment 3. Protein and carbohydrate levels vary between diets and lipid levels are constant.

Ingredients (g/kg dry weight)	Diet (% Protein) 0	15	30	45
Herring meal	---	143.4	286.8	430.2
Euphausids (freeze-dried)	---	20.7	41.5	62.7
Soybean meal	---	60.3	120.7	181.1
Wheat midlings	---	23.3	46.6	69.9
α-cellulose	61.2	39.4	19.7	---
Dextrin	285.5	193.8	102.1	10.5
Cerelose (D-glucose)	285.5	193.8	102.1	10.5
Herring oil (stabilized)[1]	180.0	158.6	137.3	115.9
Vitamin supplement[2]	30.0	30.0	30.0	30.0
Mineral supplement[3]	66.9	66.9	66.9	66.9
Carboxymethyl cellulose	14.3	14.3	14.3	14.3
Choline chloride (60%)	5.8	5.8	5.8	5.8
Ascorbic acid	2.0	2.0	2.0	2.0
DL-methionine	---	0.28	0.57	0.85
Calcium phosphate dibasic	52.3	35.9	17.9	---
Calcium carbonate	16.6	11.4	5.7	---
Estimated lipid (% dry matter)	18.0	18.0	18.0	18.0
Estimated metabolizable energy[4] (kcal/kg)	3700	3700	3700	3700

[1]Stabilized by adding 0.5g ethoxyquin/kg oil.
[2]The vitamin supplement supplied the following levels of nutrients/kg dry diet: Vitamin A acetate, 10 000 IU; cholecalciferol (D_3), 2400 IU DL-alpha-tocopheryl acetate (E), 600 IU; menadione, 26 mg; D-calcium pantothenate, 193 mg; pyridoxine HCl, 44.9 mg; riboflavin, 60 mg; niacin, 300 mg; folic acid, 20 mg; thiamine mononitrate, 36.3 mg; biotin, 3 mg; cyanocobalamin (B_{12}), 0.06 mg; inositol, 400 mg.
[3]See Appendix 3.
[4]Calculated according to Beamish et al (1986).

RESULTS

Experiment 1:

There was no significant effect of lipid/CHO composition on either K_m or V_{max} values. Dietary T_3 caused a progressive decrease in V_{max} of 5'D from a mean of 3.3 pmol T_4 conv'd/h/mg protein at 0 ppm T_3 to a mean of 0.6 at 12 ppm T_3, with no change in K_m (Table 13). Final weights of fish are also given in Table 13.

Experiment 2:

There were no significant differences in K_m at Week 5. The V_{max} values were significantly different between all groups at 5 weeks. Greatest to least values were as follows: 44%>37%>30%>23>44%(0.68%)>44%(0.52%) (Table 14). Final weights of fish are also given in Table 14.

Experiment 3:

There were no significant differences in K_m at Week 5. The V_{max} values were significantly different among all groups at 5 weeks. Greatest to least values were as follows: 45%>30%>15%>45%(0.67%)>45%(0.33%)>0% (Table 15). Final weights of fish are also given in Table 15.

DISCUSSION

Activity of 5'D was not altered significantly by the lipid/CHO ratio in the food of Experiment 1. The total and

Table 13. Effect of diet composition and T_3 supplementation on V_{max} and K_m of the hepatic 5'D system in Experiment 1 (varying lipid and carbohydrate levels). Each value represents the mean of 3 replicate measurements conducted on each of two separate pools of livers. Final body weights of fish on the various diets are given in column 5 (n = 20).

Added T_3 (ppm)	Diet	V_{max} (pmol T_4 conv'd/ h/mg prot)		K_m (X 10^{-9} M)		Final body weight (g)
0	HL	3.7	a[1]	0.9	a[1]	100.6
	ML	3.1		1.3		103.1
	LL	3.1		1.0		86.3
4	HL	1.8	b	1.2	a	111.2
	ML	2.0		1.4		100.7
	LL	1.6		1.0		84.1
8	HL	1.0	b,c	1.4	a	110.7
	ML	1.5		1.4		116.5
	LL	0.8		1.4		91.8
12	HL	0.7	c	1.4	a	115.5
	ML	0.7		1.0		105.4
	LL	0.4		1.6		95.6

[1]There was no effect of diet on V_{max} or K_m, but there was a significant effect of added T_3 on V_{max}. Within any given column, groups of values with similar letters do not differ significantly.

Table 14. Effect of diet composition on V_{max} and K_m of the hepatic 5'D system in Experiment 2 (varying protein and lipid levels). Each value represents the $\bar{X} \pm$ S.E.M. n = 2. Final body weights of fish on the various diets are given in column 4 (n = 20).

Diet (% protein)	V_max (pmol T_4 conv'd/ h/mg prot)	K_m (X 10^{-9} M)	Final body weight (g)
44	$4.18 \pm 0.14^{a,1}$	1.67 ± 0.20^{a}	62.6 ± 1.4^{a}
37	3.11 ± 0.085^{b}	1.13 ± 0.090^{a}	57.7 ± 1.2^{abc}
30	2.38 ± 0.085^{c}	1.05 ± 0.090^{a}	56.7 ± 1.5^{bc}
23	2.00 ± 0.105^{d}	1.09 ± 0.090^{a}	60.7 ± 1.7^{ab}
44 (68% ration)[2]	1.85 ± 0.025^{e}	1.27 ± 0.030^{a}	56.3 ± 1.1^{bc}
44 (52% ration)[3]	1.65 ± 0.065^{f}	1.09 ± 0.025^{a}	53.6 ± 1.6^{c}

[1]Different letters differ significantly ($p<0.05$).
[2]Protein equivalent to 30% diet, but only 68% of calories.
[3]Protein equivalent to 23% diet, but only 52% of calories.

Table 15. Effect of diet composition on V_{max} and K_m of the hepatic 5'D system in Experiment 3 (varying protein and carbohydrate levels). Each value represents the $\bar{X} \pm$ S.E.M. n = 2. Final body weights of fish on the various diets are given in column 4 (n = 20).

Diet (% protein)	V_max (pmol T_4 conv'd/ h/mg prot)	K_m (X 10^{-9} M)	Final body weight (g)
45	5.38 ± 0.225[a,1]	1.11 ± 0.060[a]	62.2 ± 1.7[a]
30	4.58 ± 0.075[b]	1.04 ± 0.085[a]	61.7 ± 1.3[a]
15	3.52 ± 0.105[c]	1.12 ± 0.042[a]	54.4 ± 1.1[b]
0	1.84 ± 0.055[d]	1.11 ± 0.030[a]	47.1 ± 1.1[c]
45 (67% ration)[2]	2.88 ± 0.020[e]	1.09 ± 0.020[a]	57.3 ± 1.0[ab]
45 (33% ration)[3]	2.33 ± 0.065[f]	1.10 ± 0.025[a]	52.6 ± 1.1[b]

[1]Different letters differ significantly ($p<0.05$).
[2]Protein equivalent to 30% diet, but only 67% of calories.
[3]Protein equivalent to 15% diet, but only 33% of calories.

free plasma levels of T_4 and T_3 taken from fish used in this study (Eales et al, 1990) were uninfluenced by dietary lipid/CHO ratio at 6.5°C. Hilton et al (1988) also found that the lipid/CHO ratio did not alter plasma T_3/T_4 levels of trout held at 15°C. Results from Experiments 2 and 3 demonstrate that protein is the most important factor in the diet for maintenance of high levels of 5'D. Total calories also seem to be important, as 5'D was less active in diets with equal protein levels but less calories (Experiment 2), although the 0% protein diet, even with the full caloric supplement, was the most effective in reducing 5'D activity in Experiment 3.

This study indicates that protein affects salmonid 5'D, but in both birds and mammals protein does not have the same effect on plasma T_3 or 5'D. Caloric intake affects T_3 production in birds (Piekarzewska et al, 1987; Rosebrough, et al, 1989) and mammals (Curran-Celento, 1987; Jennings et al, 1979; Heinen et al, 1981; Senga et al, 1982). However, high protein levels seem to decrease, rather than increase, T_3 production in birds (Pitman et al, 1986; Keagy et al, 1987; Fancher and Jensen, 1988), and consumption of a low protein diet is associated in rats with a marked rise in serum T_3 levels (Tulp et al, 1979; Rothwell et al, 1982).

In mammals, a low protein diet causes an elevation in hepatic mitochondrial α-glycerol phosphate dehydrogenase activity (Rothwell et al, 1983; Tyzbir et al, 1981), increased

heat production and reduced efficiency of energy retention (Kevonian et al, 1984). Hillgarten and Ramsos (1987) refuted the possible link between circulating low protein levels, T_3 levels and adaptive thermogenesis, but were unable to rule out a permissive role of T_3 in reducing efficiency of energy retention. In chicks, elevations in T_3 and thyroid controlled α-glycerol-3-phosphate dehydrogenase occur due to protein deficiency, but there is no link to increased thermogenesis because of the apparent lack of brown adipose tissue in chicks (Keagy et al, 1987). It is apparent in the present study on rainbow trout that increased protein levels do not lead to a decreased production of T_3. Links between protein intake, T_3 levels and cellular metabolism in fish await further study.

In rats, CHO appears to be the most crucial diet constituent for unimpaired T_3 production. Pasquali et al (1982) looked at the effects of different hypocaloric and CHO deficient diets, and concluded that (i) lowered CHO levels decreased plasma T_3; (ii) the CHO content of the diet was of greater importance that non-CHO content in modulating peripheral thyroid hormone metabolism; and (iii) the influence of total calories was perhaps as pronounced as that of CHO when a permissive amount of CHO was ingested. Hepatic deiodinase in rats responds to less than 3g of glucose refeeding after starvation (Senga et al, 1982), and Gavin et al (1980) have shown that glucose, more than protein or total calories, is necessary for normal T_4 to T_3 conversion. In

humans, like rats, CHO and dietary fat appear to be the most important dietary constituents for promoting T_3 production (O'Brien, et al, 1978; Otten et al, 1980), but there are indications that the influence of non-CHO calories may actually be more pronounced than those of CHO when at least a normal amount of CHO is ingested (Davidson and Chopra, 1979).

Gavin et al (1981) surmised that in mammals, protein feeding increases the sulfhydryl cofactor, whereas glucose feeding increases the hepatic content of active enzyme. Later studies, howevever, indicated that glucose-evoked recovery of 5'D activity may be independent of de novo protein synthesis (Nishida and Pittman, 1990).

For carnivorous fish, such as salmonids, protein and lipid are the most essential parts of the diet (Cho and Kaushik, 1990). Salmonids have a highly developed capacity to digest protein and to utilize it as an energy source (Smith et al, 1978) and a limited ability to digest CHO (Cowey and Luquet, 1983). The importance of protein levels, rather than CHO levels, in influencing 5'D activity in rainbow trout, is perhaps therefore related to the type of digestible energy available to them.

The effect of exogenous T_3 was to lower the V_{max} for the hepatic 5'D, indicating a decrease in the functional level of the hepatic deiodinase enzyme. A probable explanation is that less deiodinase is present and that, under the conditions of T_3 excess, the endogenous production of T_3 has been reduced,

perhaps by either a decrease in the level of production, or an increase in the degradation rate of the enzyme. Such autoregulation has been demonstrated previously *in vivo* in rainbow trout following acute loading with T_4 (Fok and Eales, 1984). Follow-up studies similar in nature to this experiment have indicated that T_3 5'D activity can be impaired even after T_3 supplementation is stopped, and that a single meal with 12 ppm T_3 decreases the V_{max} of 5'D (Finnson and Eales, pers. commun.). Studies are presently under way in this laboratory to examine the interaction of T_3, 5'D, diet, and growth.

In mammals, thyroid hormones influence 5'D activity. Type I 5'D responds to hypothyroidism by a decrease in activity, and to hyperthyroidism by an increase in activity. Type II 5'D responds in a reverse fashion to hypo- and hyperthyroidism (Silva and Larsen, 1986). This pattern of response to thyroid hormones occurs not only *in vivo*, but *in vitro* as well. T_3 and T_4 induce Type I 5'D activity in cultured fetal mouse tissue (Han *et al*, 1986). Addition of T_3 and T_4 to fetal rat brain culture (Leonard *et al*, 1990) and mouse neouroblastoma cells (St. Germain, 1986) causes a decrease in Type II 5'D activity, although T_3 is less effective than T_4. The effect of T_3 on the high K_m (Type I) mammalian enzyme, which corresponds closely in characteristics to the low K_m trout 5'D (Chapter 1), is opposite to the effect which T_3 has on the trout 5'D enzyme studied here. As more work is carried out on the 5'D systems in mammals and fish,

the physiological reasons for differences and similarities between the two groups as regards control of 5'D activity, will perhaps become clearer.

In conclusion, the present study has demonstrated that protein is the most important diet constituent for long-term activation of 5'D activity through an increased amount of functional deiodinase (V_{max}). Addition of T_3 into the diet decreases the V_{max} of 5'D, suggesting a reduction in the amount of functional enzyme.

CHAPTER 5

THE SOURCE OF T_3 SATURABLY BOUND TO T_3 NUCLEAR RECEPTORS IN VARIOUS TISSUES

INTRODUCTION

Studies on the deiodinase enzyme in rainbow trout have described enzymatic activity in the liver (Leatherland, 1981; Shields and Eales, 1986; MacLatchy and Eales, 1988; MacLatchy and Eales, 1990), kidney (Leatherland, 1981; Chapter 1 this study), gill (Chapter 1 this study) and muscle (Chapter 1 this study). T_3 produced from T_4 is believed to bind to nuclear T_3 receptors thereafter altering chromosomal gene expression and eliciting changes in mRNA concentration, and eventually influencing cellular metabolism (Oppenheimer, 1983). The T_3 that binds to nuclear receptors within a particular tissue has two possible sources. The T_3 could have been derived intracellularly; or the T_3 could have been part of the plasma T_3 pool prior to coming into the tissue and binding to nuclear receptors.

The central questions investigated in this chapter are: What is the source of the T_3 which is saturably bound to nuclear receptors in fish tissues? Is it derived from from intracellular 5'D activity or from the plasma T_3 pool? Does the source of receptor-bound T_3 vary from tissue to tissue? In the rat, 78% of nuclear T_3 receptor sites in the anterior pituitary were occupied by T_3 (Silva et al, 1978). Half of

this T_3 was derived directly from the plasma T_3 pool and the other half from intracellular generation by T_4 deiodination. Local T_4 monodeiodination provided only 28% and 14%, respectively, of the nuclear T_3 in liver and kidney, in which tissues the nuclear receptors were about 50% saturated (Silva et al, 1978). That study was carried out by labelling plasma T_3 and intracellular T_3 with two different iodine isotopes (^{125}I and ^{131}I). A similar approach was used in this study on rainbow trout.

Previous studies on the effects of various physiological and endocrine factors on 5'D in salmonids have concentrated on changes in the activity of the hepatic enzyme (Shields and Eales, 1986; Cyr et al, 1988b; MacLatchy and Eales, 1988; Vijayan et al, 1988; MacLatchy and Eales, 1990), on the assumption that these changes could be responsible for concurrent changes in plasma T_3 levels. However, without knowing the source of the T_3 occupying the nuclear receptors it is difficult to evaluate the influence of changes in plasma T_3 on the availability of T_3 for nuclear occupancy within a specific tissue. In this study we have attempted to determine for selected tissues the extent to which T_3 occupancy of nuclear receptors depends on T_3 from the plasma pool or T_3 generated from T_4 in the cell itself. This study is important for assessing the relevance of the 5'D systems in rainbow trout in generating T_3 for use in the cell or for release to the plasma T_3 pool.

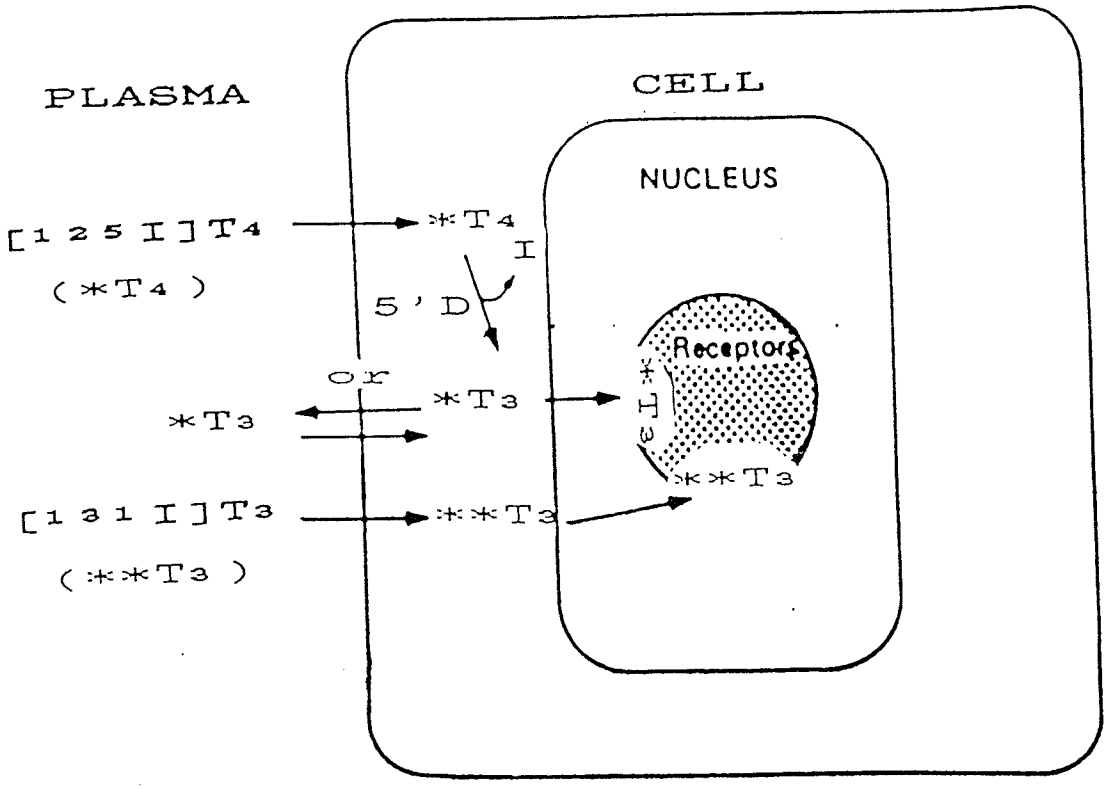
MATERIALS AND METHODS

General Theory:

The method used in this chapter is based on the procedure of Silva et al (1978). Both [^{125}I]T_4 and [^{131}I]T_3 were simultaneously injected into individual rainbow trout. The [^{131}I]T_3, when bound to nuclear receptors, represents the T_3 derived from the plasma pool. The [^{125}I]T_3 that is bound to nuclear receptors represents T_3 that was produced by conversion of T_4 to T_3 by 5'D intracellularly. Taken into consideration in the calculations is a correction for [^{125}I]T_3 generated inside cells, which could travel to the plasma pool and then be available for cellular uptake and binding to nuclear receptors in other cells or tissues (see Results, Sample Calculation, Line 6). The fish were sampled 20 h after the injection of labelled hormones, when a steady state had been reached between the labelled hormones in the plasma and labelled T_3 bound to the nuclear receptors (Appendix 4; Bres, 1987). Of the T_3 bound to the nucleus, only saturably bound T_3 was considered. When the previous conditions are satisfied, the ratio of ^{125}I/^{131}I which is saturably bound provides an index of the nuclear T_3 receptors occupied by intracellular T_3. Figure 21 is a schematic representation of the theory upon which this method is based.

Fish Maintenance:

Figure 21. Diagramatic representation of dual-labelled thyroid hormone experiment to determine the source of T_3 bound to nuclear receptors. [^{125}I]T_4 ($*T_4$) and [^{131}I]T_3 ($**T_3$) are injected into rainbow trout and travel from the plasma into the cell. $*T_4$ is converted into $*T_3$ by 5'D. $*T_3$ then either binds to nuclear receptors within the cell, or travels back into the plasma from which it can potentially re-enter other cells. $**_{T3}$ can bind immediately to nuclear receptors upon entering the cell.


PLASMA
CELL
NUCLEUS
[125I]T4
(*T4)
*T4
I
5'D
or
*T3
*T3
Receptors
[I3]
[131I]T3
(**T3)
**T3
**T3

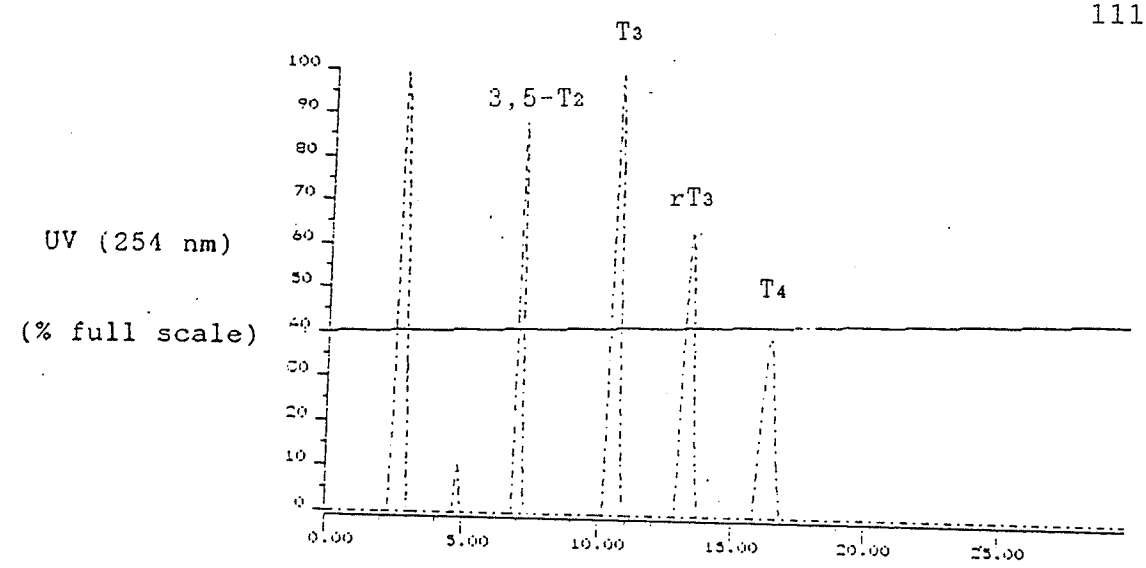
Immature rainbow trout were obtained from the Rockwood Hatchery, Balmoral, Manitoba, and held in tanks with continuosly flowing dechlorinated Winnipeg city water at 12°C under a constant photoperiod (12-h L : 12-h D). Fish were fed 2% of body weight (Trout Grower Pellets, Martin Feed Mills, Ontario) each morning and were acclimated for 6 weeks prior to the start of the experiment.

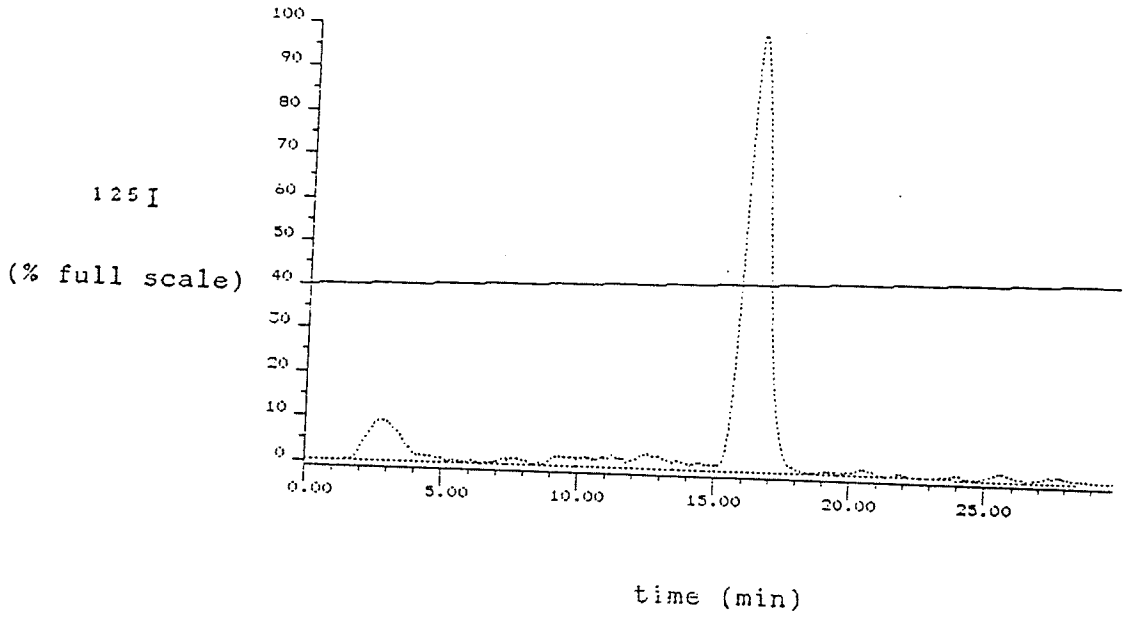
Preparation of labelled hormones:

Radioactively labelled T_4 ($[^{125}I]$ T_4) was obtained from Amersham International, and was purified using a method developed by R. Sweeting (Appendix 5). Purity of the T_4 was determined by HPLC (see following HPLC methodology). The purified T_4 contained 7.02% $^{125}I^-$ at the time of use in the experiment (Figure 22).

Radioactively labelled $[^{13}{}_1I]$ T_3 was produced in the laboratory according to the chloramine-T method of Weeke and Orskov (1973). To 2 mCi ^{131}I was added 50 uL of 50 mM NaH_2PO_4 buffer, pH 7.5. Thereafter, 2 ug (20 µL) of 3,5-diiodo-L-thyronine (Sigma) in buffer and 90 ug (25 µL) of chloramine-T in buffer were added. The reaction was halted with 240 ug (100 µL) sodium metabisulphite in water after 15 s. The reaction mixture was separated by adsorption chromatography using a 5-mL Quick-Sep Column (Isolab, Inc., OH) containing 0.25 g of LH-20 Sephadex. The column was first eluted with 2 mL 0.1N HCl, followed by 8 mL of H_2O. One-millitre aliquots of 1:9 methanol : 0.1N NaOH were then eluted and collected. The 6th,

Figure 22. HPLC chromatograms for determination of purity of [^{125}I]T_4 for injection into rainbow trout. Figure 22A is the chromatogram of authentic, unlabeled, UV-absorbing standards; Figure 22B is the chromatogram of injected [^{125}I]T_4. Injected [^{125}I]T_4 contained 7.04% labeled radioiodide contamination.


T3
3,5-T2
rT3
T4
UV (254 nm)
(% full scale)
100
90
80
70
60
50
40
30
20
10
0
0.00
5.00
10.00
15.00
20.00
25.00
time (min)


125I
(% full scale)
100
90
80
70
60
50
40
30
20
10
0
0.00
5.00
10.00
15.00
20.00
25.00
time (min)

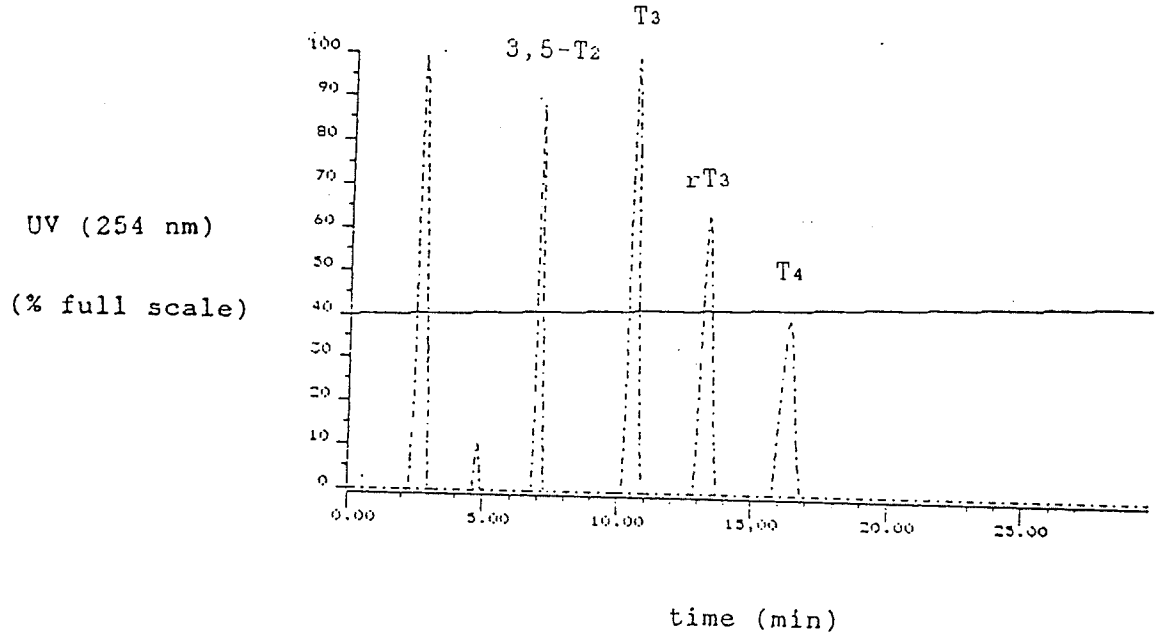
7th and 8th elutions contained the T_3, as separated by HPLC (Figure 23). The fractions were neutralized by 0.1 N HCl, and dissolved in 20 µL aqueous propylene glycol (50:50; v/v) for injection.

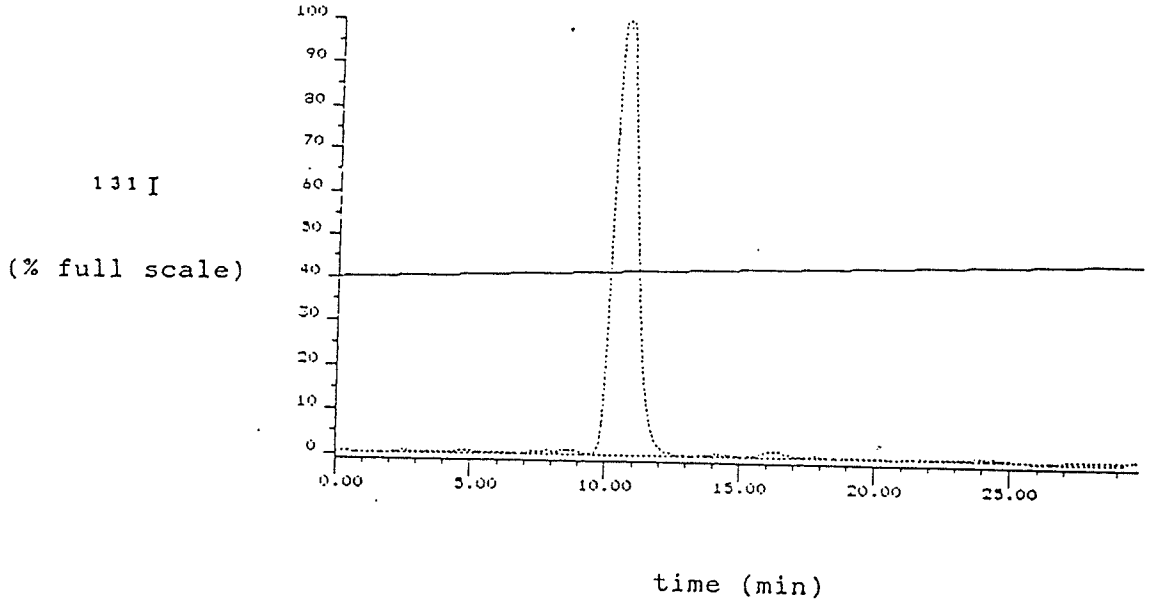
HPLC Analysis:

HPLC analyses were conducted using a Gilson IBM binary gradient system. The solvent system used was acetonitrile (0.1% trifluoroacetic acid)/water (0.1% trifluoroacetic acid), with the acetonitrile gradient increasing linearly from 40-45% (0-30 min) (Brown et al, 1990). The solvent was HPLC grade and was filtered and degassed before use.

Samples of 50 µL were injected into the system and iodothyronine separation achieved by reverse phase at 30°C at a flow of 1 mL/min on a 150-mm X 4.6-mm C_{18} (0.5 µL) Alltech Econosphere column (#70060) with a C_{18} guard column (Brown et al, 1990). Synthetic iodothyronine standards (T_2 = 3,3'-diiodo-L-thyronine; T_3 = 3,3,5'-triiodo-L-thyronine; rT_3 = 3,3',5'-triiodo-L-thyronine and T_4 = L-thyroxine; Sigma or Henning Berlin, GMbH/Berlin, Germany) were dissolved in methanol and either added to sample vials so that they could be detected simultaneously with the ^{125}I-labelled derivatives, or run at regular intervals between samples. Following elution from the column the solvents passed through a UV detector (254 nm; cell volume 12 µL), then through a Ramona 90 gamma radiation spectrometer with automatic background subtract (window optimized for ^{125}I counting; cell volume 450 µL) and

Figure 23. HPLC chromatograms for determination of purity of [^{131}I]T_3 for injection into rainbow trout. Figure 23A is the chromatogram of authentic, unlabeled, UV-absorbing standards; Figure 23B is the chromatogram of injected [^{131}I]T_3.


UV (254 nm)
(% full scale)
T3
3,5-T2
rT3
T4
100
90
80
70
60
50
40
30
20
10
0
0.00
5.00
10.00
15.00
20.00
25.00
time (min)


131 I
(% full scale)
100
90
80
70
60
50
40
30
20
10
0
0.00
5.00
10.00
15.00
20.00
25.00
time (min)

then either to waste or to a Gilson Model 202 fraction collector. Data from the UV detector and gamma detector were fed, with programmed allowance for delay between UV and gamma detection, to an IBM computer for storage and subsequent analysis, integration and display using the Gilson 714 software package. For the current qualitative analysis the UV absorption profiles have been expressed in arbitrary units of absorption. The ^{125}I profiles were expressed as a percentage of the tallest peak recorded on each chromatogram.

Injection Protocol:

At 1230 h, designated fish were injected ip with either 1.5 X 10^6 cpm [^{131}I]T_3 and 2.7 X 10^6 cpm [^{125}I]T_4 or 0.86 X 10^6 cpm [^{131}I]T_3 and 1.3 X 10^6 cpm [^{125}I]T_4. Prior to injection of radioisotopes, 500 µL of blood was withdrawn for determination of the plasma T_3 and T_4 levels by RIA (see Chapter 2 for method). Fish were sampled 20 h later at 0830 h the next morning. Eighteen to 20 h post-injection is considered the equilibrium time point in rainbow trout, when [^{125}I]T_3 which has been injected ip at 12°C is entering and leaving the nuclei of the tissues under study at the same rate, and the specific activity of T_3 is theoretically the same in the plasma and the nuclei (Appendix 4; Bres, 1987).

Plasma levels of labelled thyroid hormones:

At sampling, fish were weighed and blood was removed from the caudal vessels using a heparinized syringe. Plasma was separated by centrifugation and stored at -70°C. In

preparation for HPLC analysis, plasma was extracted in 3 mL of of methanol, let sit for 2 h, centrifuged for 10 min at 13,000 g, and the supernatant aspirated. Plasma levels of radioactively labelled components were then separated by HPLC.

The values ultimately required for the calculations were the amounts of labelled iodine and/or thyroid hormones expressed as a percentage of the respective injected ^{125}I-labelled or ^{131}I-labelled thyroid hormones.

Nuclear binding of labelled T_3:

The method of isolating and purifying nuclei was based on that used by Bres and Eales (1986). After removal of blood, the trout were killed by a blow on the head. Previous studies by Bres (1987), and myself, have shown that attempts to isolate fish muscle nuclei for the measurement of saturable binding based on currently available methods are unreliable, and muscle tissue was therefore not used in this study. The remaining tissues of interest, liver, gill filaments, and kidney were removed, and placed in ice-cold buffer A (0.32 M sucrose, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.6, 1 mM DTT, 1 mM spermidine), weighed and minced with scissors in 4 volumes of buffer A. These and subsequent steps were carried out on ice. Liver and kidney were homogenized (Polytron) for 3 s, followed by a two-stroke homogenization with a Potter-Elevhjem homogenizer with a motor-driven pestle. The gill tissue was homogenized with the Polytron 4 times for 15 s, with 20 s cooling periods in between. Liver and kidney homogenate were

then filtered through 4 layers of cheesecloth; gill was filtered through 8 layers. Following this, the homogenates were diluted with buffer A to 8 volumes (w/v). The filtrate was centrifuged for 10 min at 18,800g at 4°C to produce a crude nuclear peller. The pellet was resuspended in 8 volumes of buffer B (2.3 M sucrose, 3 mM $MgCl_2$, 1mM DTT, 1 mM spermidine, 10 mM Tris-HCl, pH 7.5), and centrifuged at 125,000g for 1 h using a Beckman SWTi40 swinging-bucket rotor. The purified nuclear pellet thus obtained was resuspended in assay buffer AB [0.32 M sucrose, 3 mM DTT, 3 mM $MgCl_2$, 25 mM KCl, 2 nM EDTA, 0.5 mM spermidine, 20 mM Tris-HCl, pH 7.2, 5% glycerol (v/v)], and rapidly frozen in 10-mL aliquots in liquid nitrogen and then stored at -75°C for less that 24 h. On the day of use the nuclear suspension in AB was centrifuged for 8 min at 0-4°C. The pellet was resuspended in AB containing 0.25% Triton X-100 detergent, kept at 0°C for 10 min, then centrifuged as before, washed once in AB, and finally resuspended in AB at a concentration of approximately 1 g liver equivalent/ml AB.

For the T_3 binding assay, 500 µL of the nuclear suspension was added to AB to a final volume of 1 mL, and incubated for 4 h in a waterbath at 12°C with agitation. Parallel tubes with 10^{-6} M unlabelled T_3 (Sigma) were also incubated in order to determine the extent of nonsaturable binding. The incubation was ended by placing the reaction tubes on ice and adding 2 mL ice-cold AB containing 0.5%

Triton X-100. The diluted suspension was left 10 min, transferred to different tubes, and centrifuged at 1000g for 20 min at 0-4°C. Both total and nonsaturable uptake were determined in triplicate for each experimental treatment. Saturable binding was determined from the difference between those two values, and for the initial calculations only the amount of label, expressed as a percentage of the initial dose of labelled thyroid hormones was required.

The DNA content of the nuclei was measured using the method of Burton (1956). One-millilitre aliquots of thawed nuclear preparation were mixed with 8 mL of $HClO_4$ and placed on ice for 15 min. These samples were then centrifuged at 10,000g for 10 min at 0-4°C. The supernatant was then discarded, and the procedure was repeated on the pellet. Four millilitres of 0.5N $HClO_4$ were added to the pellet, and placed in a waterbath at 75°C for 15 min. The samples were cooled and centrifuged as previous. The supernatant was collected and the high temperature extraction was repeated with 3.5 mL of 0.5N $HClO_4$. One-millitre aliquots were used for DNA determination. Standards were made (0-100 µg/mL DNA), from a stock calf thymus DNA (Sigma) solution (4 mg DNA/mL in 0.005 M NaOH, heated twice for 15 min at 75°C, 0.5 N $HCLO_4$ used to dilute stock). One-millitre aliquots of DNA extracts and standards were vortexed with 2 mL of fresh diphenylamine reagent (1.5 g diphenylamine, 100 mL glacial acetic acid, 1.5 mL concentrated HCl, 8.12 mg aqueous acetaldehyde). The DNA-

diphenylamine solutions were incubated for 20 h at 30°C in a shaking waterbath. DNA was measured at 595 nm.

RESULTS

The following results were obtained from the procedures used in this experiment:

Plasma T_3 and T_4 levels: Plasma T_3 and T_4 levels taken before injection of radioactive thyroid hormones are given in Table 16.

Plasma levels of $[^{131}I]T_3$: Plasma levels of $[^{131}I]T_3$ at 20 h are listed in Table 17.

Plasma levels of total hormonal ^{125}I: Plasma levels of total hormonal ^{125}I at 20 h are listed in Table 18.

Plasma levels of $[^{125}I]T_3$: Plasma levels of $[^{125}I]T_3$ at 20 h are listed in Table 19.

Saturably bound nuclear $[^{131}I]T_3$: Values for saturably bound nuclear $[^{131}I]T_3$ are given in Table 20.

Saturably bound nuclear $[^{125}I]T_3$: Values for saturably bound nuclear $[^{125}I]T_3$ are given in Table 21.

The previous values were used to calculate the contribution of "intracellular" and "plasma-pool" T_3 to nuclear receptors in liver, gill, and kidney of the rainbow trout. A sample calculation for the liver tissue of Fish # 1 follows.

Sample calculation:

Dose: $[^{125}I]T_4$ 1.3 X 10^6 cpm

Table 16. Plasma T_3 and T_4 levels in 7 individual rainbow trout prior to injection of $[^{125}I]T_4$ and $[^{131}I]T_3$. Body weights on the final sampling day are also given.

Fish #	Body Weight (g)	Plasma T_3 (ng/mL)	Plasma T_4 (ng/mL)
1	120.6	3.1	3.3
2	142.7	2.7	3.5
3	139.8	4.1	4.9
4	162.5	6.1	6.9
5	173.1	3.8	4.7
6	154.6	4.7	4.2
7	163.9	4.9	5.8
$\bar{X}$	151.0	4.2	4.8
S.E.M.	6.8	0.44	0.48

Table 17. Plasma levels of $[^{131}I]T_3$ in 7 individual rainbow trout at the time of sampling. Counts have been corrected for radioactive decay, and adjusted for 1 mL of plasma.

Fish #	$[^{131}I]^{T}_{3}$ (cpm/mL plasma)	$[^{131}I]T_3$ (% dose/mL)
1	1722	0.231
2	1077	0.140
3	4127	0.554
4	6858	0.456
5	10116	0.673
6	14125	0.940
7	8849	0.590
$\bar{X}$	6696	0.512
S.E.M.	1789	0.102

Table 18. Plasma levels of total hormonal ^{125}I and in 7 individual fish. Counts have been corrected for ^{131}I counts appearing in the ^{125}I window, for radioactive decay, and adjusted for 1 mL of plasma.

Fish #	[^{125}I] (cpm/mL plasma)	[^{125}I] (% dose/mL)
1	6310	0.702
2	4192	0.467
3	276	0.092
4	4367	0.727
5	7033	1.270
6	4165	0.693
7	1861	0.621
$\bar{X}$	4029	0.653
S.E.M.	889	0.133

Table 19. Plasma levels of [^{125}I]T_3 in 7 individual fish. Counts have been corrected for ^{131}I counts appearing in the ^{125}I window, for radioactive decay, and adjusted for 1 mL of plasma. Values for percent dose/mL represent plasma [^{125}I]T_3 as a percentage of the injected dose.

Fish #	[^{125}I]T_3 (cpm/mL plasma)	[^{125}I]T_3 (% dose/mL)
1	374	0.042
2	109	0.012
3	20	0.003
4	262	0.015
5	864	0.048
6	456	0.025
7	173	0.096
$\overline{X}$	323	0.034
S.E.M.	107	0.012

Table 20. Saturable binding of nuclear T_3 receptors with $[^{131}I]T_3$ in 7 individual fish. Counts have been corrected for radioactive decay, and for ^{131}I counts appearing in the ^{125}I window. Values for % of dose represent saturably bound $[^{131}I]T_3$ as a percentage of the injected dose.

Tissue	Fish #	Saturably bound $[^{131}]T_3$ (% dose) ($X\ 10^{-4}$)	Saturably bound $[^{131}I]T_3$ (% dose/ug DNA) ($X\ 10^{-4}$)
Liver	1	7.2	4.1
	2	4.1	3.1
	3	6.6	5.3
	4	4.3	2.2
	5	13.3	3.7
	6	20.1	4.6
	7	22.8	3.3
Kidney	1	5.0	7.2
	2	2.9	6.1
	3	5.7	9.4
	4	2.7	6.4
	5	7.2	10.0
	6	5.2	12.0
	7	2.8	8.9
Gill	1	0.30	1.9
	2	0.39	1.7
	3	0.38	2.1
	4	0.15	0.45
	5	0.81	3.1
	6	0.20	0.63
	7	0.80	2.1

Table 21. Saturable binding of nuclear T_3 receptors with $[^{125}I]T_3$ in 7 individual fish. Counts have been corrected for radioactive decay. Values for % of dose represent saturably bound $[^{125}I]T_3$ as a percentage of the injected dose.

Tissue	Fish #	Saturably bound $[^{125}]T_3$ (% dose) ($X\ 10^{-4}$)	Saturably bound $[^{125}I]T_3$ (% dose/mg DNA) ($X\ 10^{-4}$)
Liver	1	7.9	4.5
	2	4.6	3.5
	3	7.3	5.9
	4	4.5	2.3
	5	13.6	3.8
	6	20.5	4.7
	7	24.2	3.5
Kidney	1	1.6	3.4
	2	1.5	2.9
	3	2.7	4.4
	4	0.97	2.3
	5	2.7	3.8
	6	1.9	4.4
	7	1.1	3.3
Gill	1	5.3	3.4
	2	1.9	8.3
	3	1.8	10.0
	4	0.88	2.4
	5	1.5	5.7
	6	1.0	3.3
	7	1.1	2.9

$[^{131}I]T_3$ 0.86 X 10^6 cpm

Plasma T_3 3.1 ng/mL

Plasma T_4 3.3 ng/mL

Nuclear DNA/liver (mg) 1.75 X 10^{-2}

Nuclei and plasma samples are counted simultaneously and suitable corrections are performed for ^{131}I counts appearing in the ^{125}I spectrometer window, for radioactive decay. All results are then expressed as a fraction of the injected dose which was counted simultaneously. The percentage of the injected dose, as measured in the plasma or the nucleii, is the common unit for all calculations.

For $[^{131}I]T_3$:

1. Nuclear $[^{131}I]T_3$/liver (percentage of dose/mg DNA):

4.1×10^{-4}

2. Plasma $[^{131}I]T_3$ (percentage of dose/mL): 0.231

3. Nuclear to plasma ratio (line 1/line 2): 1.78 X 10^{-3}

For $[^{125}I]T_4$:

4. Nuclear $[^{125}I]T_3$ (percentage of dose/mg DNA):

4.5×10^{-4}

5. Plasma total hormonal ^{125}I (percentage of dose/mL): 0.702

6. $[^{125}I]T_3$ in plasma (percentage of dose/mL): 0.042

7. Nuclear $[^{125}I]T_3$ from plasma $[^{125}I]T_4$ (line 3 X line 6):

7.4×10^{-5}

8. Locally generated nuclear $[^{125}I]T_3$ (line 4 - line 7):

3.8 X 10-^{4}

9. Total locally generated nuclear [^{125}I]T_3 (line 8 X 2; this corrects for loss of ^{125}I from T_4 during monodeiodination to T_3): 7.5×10^{-4}

10. Total locally generated nuclear [^{125}I]T_3 to plasma T_4 ratio (line 9/line 5): 1.1×10^{-3}

11. Nuclear T_3 from plasma T_3 (line 3 X plasma T_3): 5.4×10^{-3}

12. Nuclear T_3 from cellular T_3 (line 10 X plasma T_4 X (651/777)): 2.8×10^{-3}

13. Percentage of saturably-bound T_3 derived from intracellular T_3 (line 11/(line 11 + line 12) X 100): 34.1%

Using the above calculation for each tissue, the following results were obtained:

Nuclear T_3 from plasma T_3: The amount of nuclear T_3 from plasma T_3 (as derived from equation 11) is given in Table 22.

Nuclear T_3 from cellular T_3: The amount of nuclear T_3 from locally generated T_3 (as derived from equation 12) is given in Table 23.

Percentage of saturably-bound T_3 derived from intracellular sources: The percentage of T_3 bound to nuclear receptors from locally generated T_3 (as derived from equation 13) is given in Table 24.

Table 22. Saturably bound nuclear T_3 derived from plasma T_3 from 7 individual fish. (Based on calculation #11).

Fish #	Liver	Kidney (ng T_3/mg DNA X 10^{-3})	Gill
1	5.4	4.9	2.6
2	5.9	5.6	3.3
3	3.9	7.0	1.6
4	2.9	8.8	0.52
5	2.1	5.0	0.18
6	2.3	1.4	0.72
7	2.7	5.2	1.4
$\bar{X}$	3.6	5.4	1.5
S.E.M.	0.57	0.85	0.43

Table 23. Saturably bound nuclear T_3 derived from intracellular T_3 for 7 individual fish. (Based on calculation #12).

Fish #	Liver	Kidney (ng T_3/mg DNA X 10^{-3})	Gill
1	2.8	4.6	6.7
2	3.1	3.0	4.0
3	4.4	0.80	37.4
4	3.2	3.1	3.2
5	1.9	2.1	1.8
6	5.2	2.2	1.7
7	4.6	2.7	2.4
$\bar{X}$	3.6	2.6	8.2
S.E.M.	0.44	0.44	4.9

Table 24. Percentage of saturably bound nuclear T_3 derived from intracellular T_3 in 7 individual fish. (Based on calculation #13).

Fish #	Liver	Kidney (%)	Gill
1	34.1	48.2	72.4
2	34.6	34.7	54.8
3	52.8	10.2	96.0
4	51.8	25.8	86.0
5	48.6	29.8	90.9
6	69.7	14.0	69.6
7	62.5	34.5	63.4
$\bar{X}$	50.5	28.2	76.2
S.E.M	5.0	4.9	5.7

DISCUSSION

The results indicate that intracellular monodeiodination of T_4 to T_3 contributes to the nuclear saturably-bound T_3 fraction, but the extent of this contribution varies considerably between the different tissues of the rainbow trout. In gill tissue, most of the nuclear-bound T_3 is generated intracellularly; in kidney, most of the nuclear-bound T_3 is derived instead from the plasma pool; and in liver, nuclear receptors are occupied about equally by T_3 from both these sources.

In Chapter 1, two different types of 5'D were identified. A low K_m 5'D occurred in liver, muscle and gill tissue, while a high K_m 5'D occurred in liver and kidney tissue. In the present study, gill, which possesses only a low K_m 5'D, has a greater proportion of its receptor sites occupied by T_3 derived from intracellular monodeiodination. Kidney, which possesses only a high K_m 5'D, has the greatest proportion of its receptors sites occupied by T_3 from the plasma pool. Liver tissue, which contains both low and high K_m 5'D, has approximately equal proportions of T_3 derived from plasma and intracellular sources bound to its receptor sites. These correlations may indicate that tissues with low K_m 5'D are able to supply the nuclear receptors in that tissue with adequate amounts of T_3, without reliance on plasma pool T_3. In contrast, tissues with high K_m 5'D may rely more on the plasma pool T_3.

In the rat, the relationship between the type of 5'D present in a given tissue, and the source of nuclear-bound T_3 has a major physiological significance. The contribution of intracellularly-derived T_3 to nuclear-bound T_3 is much greater in the pituitary than in the liver and kidney (Silva et al, 1978). The rat pituitary contains the Type II, low K_m 5'D, whereas liver and kidney contain the Type I, high K_m 5'D (see Introduction, Chapter 1). Type II 5'D in rat pituitary has been linked to the tight control of the thyroid hormone status in that tissue, as there is a relationship between pituitary Type II 5'D, pituitary nuclear T_3 that was intracellularly generated, and suppression of TSH release in hypothyroid animals (Silva and Larsen, 1978). Tight regulation of T_3 nuclear occupancy in pituitary could prevent short-term hypothyroidism in the animal, because there is little reliance on plasma T_3 (Silva et al, 1978). The circumstantial evidence found in the present study on rainbow trout also suggests a relationship between the type of 5'D (low or high K_m) and the proportion of nuclear receptors occupied by locally-derived T_3, but the physiological implications of this relationship await further study.

Studies to date on factors that affect 5'D and plasma levels of T_3 in fish have centred on hepatic 5'D and concurrent changes in plasma levels of thyroid hormones. This study indicates that changes in plasma T_3 may not influence nuclear occupancy in all tissues to the same degree. The

results from this experiment were obtained from well-fed fish held under standardized laboratory conditions, and it is difficult to predict the effects that different physiological states may have on the relationship between plasma and intracellular sources of T_3. For example, physiological conditions in which plasma T_3 is low, such as starvation (Leatherland, 1982; Flood and Eales, 1983), stress (high cortisol) (Brown et al, 1984; Redding et al, 1986) or high estradiol levels (Olivereau et al, 1981) would have more drastic affects on T_3 availability to nuclear receptors in kidney than in other tissues. How this would affect the overall thyroidal status of the fish is unclear at this time.

To obtain a complete picture of the thyroidal status in fish, it may be necessary to study not only plasma levels of thyroid hormones, but also 5'D activity and nuclear receptor occupancy in all tissues. These criteria have been partially met by studies on the effects of estradiol (Cyr et al, 1988b; Bres et al, 1990) and diet (Eales et al, 1990). Those studies, however, only looked at the physiological effects on hepatic 5'D and hepatic nuclear receptors (changes in binding affinity and maximal binding capacity) and plasma thyroid hormone levels. The effect of estradiol on T_3 availability to gill nuclear receptors may not be as pronounced as its effect on T_3 availability to kidney receptors, since there appears to be less dependence by gill on nuclear T_3 from the plasma.

These results also suggest that certain tissues may

contribute more than others in supplying T_3 to the plasma T_3 pool. Since only half of the nuclear receptors in liver, and 28% of the nuclear receptors in kidney are occupied by intracellular T_3, the additional T_3 that is produced in those tissues may be destined for the plasma, rather than for immediate local receptor occupancy. This could account for findings in Chapters 2 and 3, where changes in hepatic 5'D activity and plasma T_3 levels occur concurrently in response to sex steroids and GH. It may also indicate that tissues such as kidney are incapable of supplying adequate amounts of T_3 intracellularly, and therefore must depend on the plasma as a source of T_3.

In conclusion, gill nuclear receptors are occupied by intracellular, rather than plasma T_3; kidney receptors are occupied by predominately plasma T_3; and liver receptors contain both. Factors that affect plasma T_3 will therefore have differing effects on T_3 availability for nuclear receptor occupancy in various tissues.

CHAPTER 6

SUMMARY

Since T_3 is the presumed cellularly active thyroid hormone, activity of 5'D is an important regulatory step of the thyroid hormone system. Together, the results of these various experiments, and the conclusions that can be drawn from them, advance our understanding of the 5'D system(s) in teleosts. However, many questions still remain to be answered on the role of 5'D in determining thyroidal status.

Two types of 5'D have been identified in rainbow trout. The high substrate enzyme occurs in kidney and liver, and probably operates with a sequential mechanism like that of Type II mammalian 5'D. The low substrate enzyme occurs in liver, gill and muscle, and probably operates via a ping-pong mechanism like that of Type I 5'D in mammals. Since the low substrate enzyme could operate at physiological T_3 levels, it may be the principal deiodinating reaction in fish. The proportion of intracellularly-made compared to plasma-source T_3 occupying nuclear receptors also varies between tissues. Gill nuclear receptors are occupied by mostly intracellular T_3, as compared to kidney which contains mostly plasma T_3. Liver contains equal proportions of both.

Androgens, GH and high protein levels in the diet significantly increase 5'D V_{max} and plasma T_3 levels. Estradiol and T_3 effectively decrease 5'D V_{max} and plasma T_3. The concurrent changes in V_{max} and plasma T_3 lead to the

conclusion that 5'D, at least partially, is responsible for plasma T_3 changes demonstrated in these and previous studies.

The identification of two enzyme systems operating at high and low substrate levels, and the differences between the source of T_3 for nuclear receptor occupancy in liver, kidney and gill indicate that the role of 5'D in different tissues represents a complex situation. It appears that the low substrate enzyme (present in liver, gill and muscle) may be important in producing T_3 for immediate occupancy of nuclear receptors. Gill tissue contains only low substrate 5'D, and gill nuclear T_3 receptors contain 7X as much intracellular T_3 as plasma T_3. Kidney tissue, on the other hand, which contains only high substrate 5'D, gets the majority of nuclear T_3 from the plasma. Tissues that produce T_3 for nuclear receptors intracellularly would be less dependent on plasma T_3, and more dependent on circulating levels of T_4, than are plasma-T_3-dependent tissues. Tighter control of T_3 to nuclear receptors could also possibly be made in tissues such as gill, muscle and liver that contain low K_m 5'D.

The role of liver 5'D, which contains the highest activity of low K_m 5'D/unit protein, becomes significant for tissues such as RBC and kidney which contain no low K_m 5'D. As shown by the studies on testosterone, estradiol, GH, T_3 and diet protein, hepatic 5'D is affected by numerous factors, with concurrent changes in plasma T_3. For example, androgen

and estradiol increase or decrease, respectively, the levels of 5'D activity and circulating plasma T_3. These changes would have the most profound effect on RBC and kidney as compared to gill, because of their reliance on plasma T_3. Because of the inability to measure T_3 occupancy of muscle nuclear receptors, it is impossible to state whether or not muscle behaves like gill as regards nuclear occupancy.

If tissues are to a greater or lesser extent dependent on plasma T_3, studies of effects of factors on overall thyroidal status, as regards nuclear receptor occupancy, and therefore cellular effects, may not be clearly demonstrated by studying only changes in plasma T_3 or 5'D activity. To gain a meaningful picture of thyroidal hormone status it would be necessary to measure plasma T_3 and T_4, 5'D activity in several tissues, along with levels of plasma-source and intracellulary produced T_3 occupying nuclear receptors. Changes in nuclear receptor binding affinity and maximal binding capacity only indicate actual changes in the receptors themselves, not changes in the type of T_3 (plasma or intracellular) bound to the receptors. Until studies can be completed that examine the resulting location of T_3 produced by 5'D, and its status, the true role of 5'D in regulation of thyroidal status cannot be assessed.

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APPENDIX 1

Calculation of pmol T_4 converted with varying amounts of carrier T_4. (Shields and Eales, 1986).

Maximum specific activity of $*T_4$ in the tracer:

If all T_4 in the tracer is labelled $= 2.18 \times 10^{12}$ uCi

Molecular weight of T_4 = 772 g/mol

$$\frac{2.18 \times 10^{12} \text{ uCi}}{7.72 \times 10^{8} \text{ ug}} = 2824 \text{ uCi/ug}$$

Two iodine atoms in the outer ring of T_4 can be labelled

$$2824 \times 2 = 5648 \text{ uCi/ug} = \text{maximum specific activity}$$

S.a. of the tracer used in this thesis = 1200 uCi/ug

$$\% \text{ labelled } T_4 \text{ in the tracer} = \frac{1200}{5648} = 21.2\%$$

% unlabelled T_4 in the tracer = 100% - 21.2% = 78.8%

A = amount of labelled T_4 in the tracer in ng:

$$A = \underset{a}{0.212} \times \underset{b}{\text{decay factor}} \times \frac{\overset{c}{\text{cpm } *T_4}}{\underset{d}{1.54 \times 10^{6}}} \times \underset{e}{\frac{1}{1200}} \times \underset{f}{1000}$$

a) % labelled T_4 in the tracer
b) correction factor for $*T_4$ decay (from decay chart)
c) total counts reference (TCR) of $*T_4$ corrected for $*I^-$ contamination [TCR - (cpm control X 10.4^8)]
d) cpm in 1 µCi at 70% counting efficiency
e) ug T_4 in µCi calculated from s.a. of 1200 uCi/ug
f) conversion factor for ug T_4 to ng T_4
g) volume correction factor

B = amount of unlabelled T_4 in the tracer in ng

$$B = \underset{a'}{0.788} \times \frac{\text{cpm } *T_4}{1.54 \times 10^{6}} \times \frac{1}{1200} \times 1000$$

a') % of unlabelled T_4 in the tracer

Total T_4 in the tracer (labelled and unlabelled) = A + B

APPENDIX 1 (continued)

Fraction of T_4 deiodinated:

$$\frac{(\text{cpm enzyme} - \text{cpm control}) \times 10.4 \times 2^{a}}{\text{cpm } {}^{*}T_4}$$

a) accounts for labelling geometry

Total T_4 added to the incubate:

$$[A + B + \text{carrier } T_4 \text{ in ng}] \times 1.3^{a}$$

$_a$) conversion factor for ng T_4 to pmol T_4

Sample calculation:

raw data: sample = 1456 cpm (mean of two values)
control = 834 cpm (mean of two values)
TCR = 123672 cpm
decay = 0.9439 (5 days)

$$\mathbf{A} = 0.212 \times 0.9439 \times \frac{114998.4^{*}}{1.54 \times 10^{6}} \times \frac{1}{1200} \times 1000$$

$$^{*}123672 - (834 \times 10.4)$$

$$= 1.2 \times 10^{-2} \text{ ng}$$

$$\mathbf{B} = 0.788 \times \frac{114998.4}{1.54 \times 10^{6}} \times \frac{1}{1200} \times 10000$$

$$= 4.9 \times 10^{-2} \text{ ng}$$

A + B = 6.1×10^{-2} ng
Carrier T_4 = 2.52 ng
Total T_4 = 2.569 ng

Pmol T_4 converted:

$$\frac{(1456 - 834) \times 10.4 \times 2}{114998.4} \times 2.569 \times 1.3$$

=0.376 pmol T_4 converted

Incubation time: 30 min
Mg protein in incubate: 136 mg

$$\frac{0.376 \times 2}{136} = 5.53 \times 10^{-3} \text{ pmol } T_4 \text{ converted/h/mg protein}$$

APPENDIX 2

Table 25. Characteristics of 5'-iodothyronine deiodinases in the rat.

	Type I	Type II
Deiodination site	Inner and outer ring	Outer ring
Substrate prefered	$rT_3 >> T_4 > T_3$	$T_4 > rT_3$
K_m for T_4	1 uM	1 nM
Tissue location	Liver, kidney and thyroid	CNS, BAT[a] and pituitary
Thiols	Stimulatory	Stimulatory
Kinetic mechanism	Ping-pong	Sequential
Propylthiouracil	Inhibitory	No effect

[a]CNS = cental nervous system
BAT = brown adipose tissue

Source: adapted from Leonard and Visser (1986)

APPENDIX 3

Table 26. Composition of mineral supplements for the diets of Experiment 2.

Mineral	Diet				Expected Total in each Diet (mg/kg diet)
	23% Protein	30% Protein	37% Protein	44% Protein	
	(mg/kg diet)				
Ca					
(as $Ca HPO_4$)	10614	7076	3541	---	28261
(as $Ca CO_3$)	2791	1862	928	---	
P					
(as $CaHPO_4$)	8203	5467	2736	---	17317
Co					
(as $CoCl_2.6H_2O$)	1	1	1	1	1
Cu					
(as $CuSO_4.5H_2O$)	10.3	8.8	7.4	5.9	15
Fe					
(as $FeSO_4.7H_2O$)	129	108	86	64	200
Mg					
(as $MgSO_4.7H_2O$)	955	636	319	---	2000
Mn					
(as $MnSO_4.H_2O$)	85.1	81.9	78.5	75.2	96
K					
(as K_2SO_4)	2875	1223	---	---	8300[a] 8727[b] 10377[c]
Se					
(as Na_2SeO_3)	0.24	0.20	0.15	0.11	0.39
Na					
(as NaCl)	3711	3016	2319	1623	6000
Zn					
(as $ZnSO_4.7H_2O$)	91	82.4	73.3	64.5	120
I					
(as KIO_3)	5	5	5	5	unknown
F					
(as NaF)	4.5	4.5	4.5	4.5	unknown

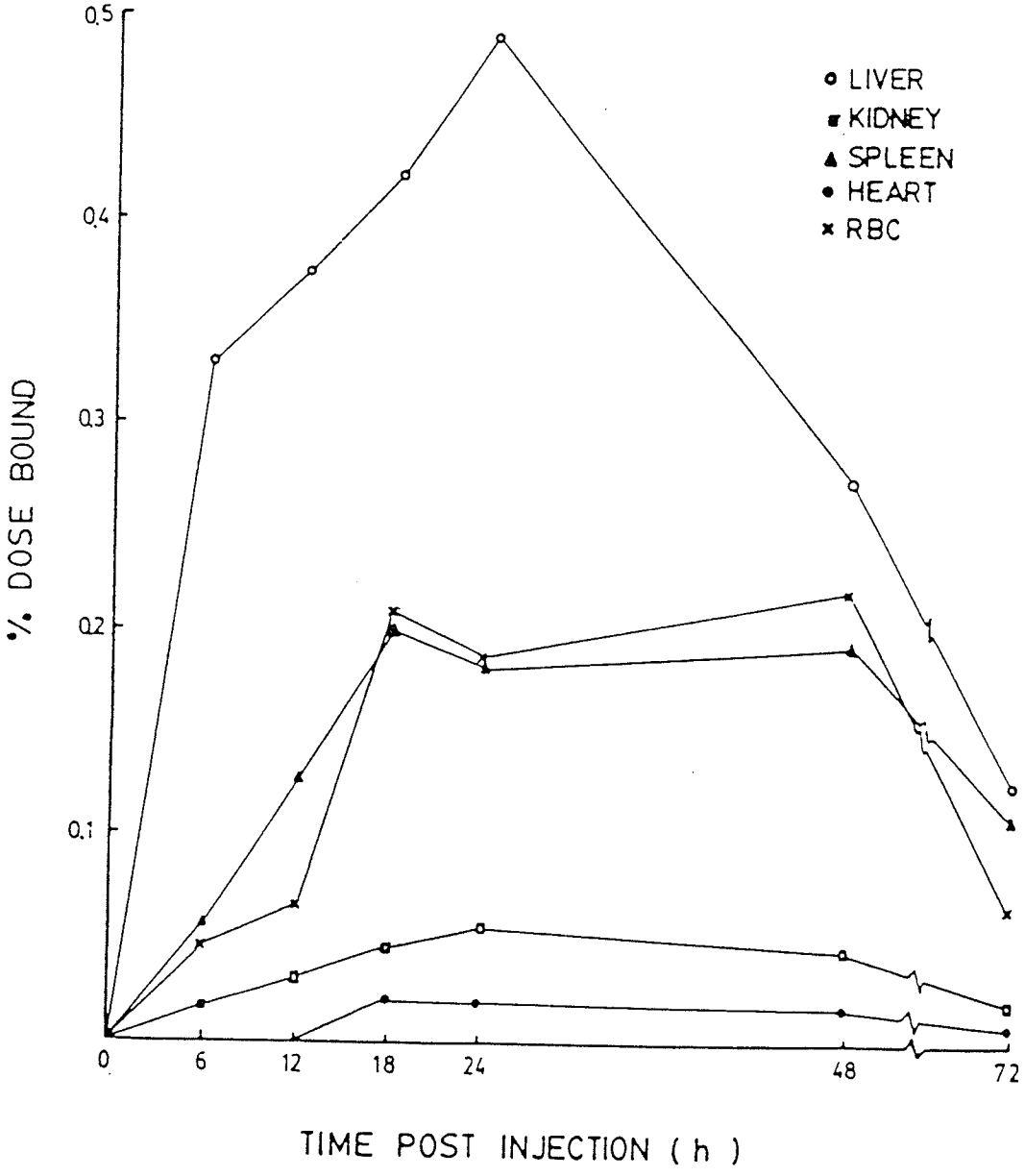
[a]23% and 30% diets
[b]37% diet
[c]44% diet

Table 27. Composition of mineral supplements for the diets of Experiment 3.

Mineral	Diet				Expected Total in each Diet (mg/kg diet)
	0% Protein	15% Protein (mg/kg diet)	30% Protein	45% Protein	
Ca					
(as $CaHPO_4$)	15399	10567	5281	---	22054
(as $CaCO_3$)	6655	4564	2282	---	
P					
(as $CaHPO_4$)	11897	8164	4083	---	14188
(as KH_2PO_4)	2291	1528	764	---	
Co					
(as $CoCl_2.6H_2O$)	1	1	1	1	1
Cu					
(as $CuSO_4.5H_2O$)	14	11.4	8.8	6.3	14
Fe					
(as $FeSO_4.7H_2O$)	200	163	125	87.8	200
Mg					
(as $MgSO_4.7H_2O$)	1802	1201	601	---	1802
Mn					
(as $MnSO_4.H_2O$)	93.1	87.1	81.1	75	93.1
K					
(as K_2SO_4)	2893	1929	964	---	8680
(as K_2CO_3)	2893	1929	964	---	
(as KH_2PO_4)	2893	1929	964	---	
Se					
(as Na_2SeO_3)	0.40	0.30	0.21	0.11	0.40
Na					
(as NaCl)	5700	4462	3224	1986	5700
Zn					
(as $ZnSO_4.7H_2O$)	120	102	84	66	120
I					
(as KIO_3)	5	5	5	5	unknown
F					
(as NaF)	4.5	4.5	4.5	4.5	unknown
Al					
(as $AlCl_3.6H_2O$)	5.4	3.6	1.8	---	5.4

APPENDIX 4

Figure 24. Time-course of saturable [^{125}I]T_3 binding to the nuclear fractions of different tissues determined in vivo. Non-saturable binding was determined in the presence of 5000 ng unlabelled T_3/100g body weight and subtracted from the total binding. Binding was not corrected for DNA recovery. Maximal binding for tissues studied was 18-24 h pi (data from Bres, 1987).


0.5
0.4
0.3
0.2
0.1
% DOSE BOUND
LIVER
KIDNEY
SPLEEN
HEART
RBC
0
6
12
18
24
48
72
TIME POST INJECTION (h)

APPENDIX 5

Method for purification of [^{125}I]T_4.

1. A 200 µL aliquot of *T_4 was pipetted atop a LH-20 Sephadex column (Isolab; 0.25 g LH-20) equilibrated with 0.1 N HCl. The column was then drained.

2. The *I^- fraction was eluted with 2 mL double distilled H_2O.

3. The purified *T_4 was collected using 3 mL of a solution containing 50% (v/v) 0.1N NH_4OH and 50% (v/v) EtOH.

4. The collected elution was then evaporated with air and heat at a low temperature, leaving the *T_4 behind.

5. The *T_4 was reconstituted in 1 mL of 0.1N NaOH. A 50 µL sample was used for purification analysis by the HPLC (see Methods, Chapter 5).