CONCENTRATION OF 3,5,3'-TRIIODO-L-THYRONINE (T_3) IN TISSUES AND ORGANS OF THE RAINBOW TROUT, SALMO GAIRDNERI

by

Pearl Y. Fok

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ABSTRACT

A method of extracting 3,5,3'-triiodo-L-thyronine (T_3) from eight tissues of rainbow trout (Salmo gairdneri) was developed. Tissues were digested enzymatically by collagenase or pronase, followed by extraction with ethanolic ammonia, and then a chloroform and ammonium hydroxide wash. Extraction efficiencies of added ^{125}I-T_3 ranged from 55.8% (kidney) to 83.8% (heart) and were determined for each tissue. However, extraction efficiencies of added unlabelled T_3 were unreliable as measured by radioimmunoassay (RIA), showing that the extraction and RIA methods were incompatible. Therefore, to determine reliably tissue T_3, ^{125}I-T_3 was administered by constant infusion to trout cannulated in the dorsal aorta until isotopic equilibrium was reached by 72h. Sephadex column chromatography was performed on tissue extracts and plasma. T_3 concentrations ($[T_3]$) were calculated using the specific activity of plasma, and the ^{125}I-T_3 content from each tissue extract. Tissues were divided into 3 groups according to T_3 concentrations. Intestine, kidney, and liver contained the most T_3, having mean concentrations of >2.0 ng/g tissue. Heart and stomach were intermediate, while gill, skin, and muscle had consistently lower concentrations of T_3. Tissue $[T_3]$ of fish stressed by exposure to acidic water (pH 4.8) or aluminum (21.6 µM) and acidic water showed similar ranking, but

absolute concentrations of T_3 were significantly lower than those of unstressed fish. Differences in tissue [T_3] to plasma [T_3] ratios between tissue types of unstressed fish were statistically significant with intestine, kidney, liver, and stomach showing ratios >1.0. Regression equations relating tissue/organ weights to body weights were used to calculate T_3 content of whole organs. For a hypothetical unstressed 300-g trout, T_3 content ranged from 0.5 ng for heart to over 100 ng T_3 for skeletal muscle. For stressed fish, T_3 content ranged from 0.31 ng for heart to 16 ng for skeletal muscle. The relatively high [T_3] in kidney, liver, and intestine, and the low muscle [T_3] are similar to literature values for rats. Tissue differences with regard to T_3 concentration and content are discussed in relation to extrathyroidal metabolism.

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INTRODUCTION

The two hormonal products secreted by the thyroid gland of mammals are L-thyroxine (T_4) and 3,5,3'-triiodo-L-thyronine (T_3). Of these thyroid hormones (TH), T_4 is released in substantially larger quantities in most mammals (Bentley 1976). T_3, however, is believed to be the principle metabolically active form of thyroid hormone at the cellular level (Oppenheimer 1979, 1983; Oppenheimer et al. 1974). Many thyroid hormone effects are initiated by the intracellular binding of T_3 to specific nuclear receptors which stimulate the synthesis of messenger RNA for protein synthesis (Kaplan 1983).

Of the T_3 available to target cells, a large majority is generated outside the thyroid gland by enzymatic 5'-monodeiodination of T_4 in peripheral tissues (Schwartz et al. 1971; Chopra 1977). The widespread existence of such deiodination pathways in extrathyroidal tissues and the much higher affinity of nuclear TH-binding sites in TH-responsive tissue for T_3 than for T_4 suggest that the question of how thyroidal status is determined depends not, as previously assumed, solely on plasma T_4 concentrations but partly on T_3 levels and T_3 interaction with the peripheral tissues (Eales 1985). The quantitative contribution of each tissue to the total production of T_3 from T_4 is largely unknown. In rats,

however, the source (i.e. plasma-derived or locally-produced in the target cell) of T_3 in parts of the brain, liver, kidney and muscle has been studied in vivo (Silva et al. 1978; van Doorn et al. 1982, 1983, 1985), and actual tissue T_3 concentrations have been documented (Nejad et al. 1975; Obregon et al. 1978; van Doorn et al. 1985).

In teleosts, the majority of work involving measurements of TH and their correlated effects has been based on blood plasma levels. The following generalizations about T_3 and T_4 plasma levels can be made (Eales 1979): (1) T_4 plasma levels in fish are lower than for mammals, (2) T_3 levels in the blood are as high or higher than for mammals, (3) T_3 plasma levels are more constant than those for T_4. It seems likely that these differences will be reflected in TH concentrations in various tissues and organs.

There has been some evidence of an increase in protein and RNA content of liver and muscle of T_3-injected tilapia, Sarotherodon mossambica (Matty et al. 1982). Also, high-affinity nuclear T_3-binding sites have been found in liver of rainbow trout, Salmo gairdneri (Van Der Kraak & Eales 1980; Bres & Eales 1986). Therefore, initiation of TH effects by intracellular T_3 binding to specific nuclear receptors may also occur in teleosts.

Furthermore, deiodination of T_4 resulting in T_3 formation has been demonstrated, at least for salmonids (Eales 1977b; Shields & Eales 1986). This conversion of TH can be expected, considering the negligible production of T_3 by the salmonid thyroid (Eales 1985). Conversion of T_4 to T_3 is of relatively greater importance as an overall source of T_3 in teleosts than it is in mammals.

Tissue concentrations of TH in fish have been based solely on tissue levels of radioactivity following single injections of labelled T_4 in Atlantic salmon, Salmo salar (Eales 1969) and in carp, Cyprinus carpio (Lone et al. 1983). In both cases, tissue radioactivity was not verified as actual T_4 but expressed as a percentage of total tissue radioactivity in relation to the injected dose of labelled T_4. Furthermore, it is doubtful if the tissues could have reached a state of isotopic equilibrium after a single injection of the hormone. Recently the T_4 content of eggs and larvae of coho salmon, Oncorhynchus kisutch (Kobuke et al. 1987) and chum salmon, Oncorhynchus keta (Tagawa & Hirano 1987) has been directly measured using radioimmunoassay (RIA). In both studies whole larvae and/or eggs were homogenized and the T_4 extracted by alcohols. Information on T_3 concentration in teleost tissues is presently not available.

Knowledge of the distribution and concentration of T_3 in teleost tissue is of fundamental importance for understanding thyroidal status and interpreting models involving compartment kinetics. A certain T_3 concentration may reflect a metabolically active or inactive tissue. Furthermore, a high concentration of T_3 possibly indicates a T_3-generating or a T_3-storage site. If a tissue is metabolically active but is known not to contribute greatly to deiodination, the presence of a high T_3 concentration may be an indication of "used" T_3, no longer bound to receptors. T_3 concentrations may also indicate possible sites of hormone degradation and/or excretion (Eales 1985).

The objectives of this study were: (1) to develop a method for the extraction of T_3 from tissues of rainbow trout, and (2) to determine T_3 concentrations in each tissue. The latter objective was attempted a) by direct measurement using radioimmunoassay (RIA) and b) by bringing the fish to isotopic equilibrium (IE) by constant infusion of [^{125}I]T_3 (*T_3) via a cannula, and then determining tissue T_3 levels from the *T_3 specific activity. Tissue values were then compared between small groups of available fish that were either unstressed or had been exposed to acid or acid and aluminum stress.

MATERIALS AND METHODS

A. Fish Maintenance and Treatment

1. Experimental Fish

Rainbow trout of both sexes and varied degrees of maturity were obtained from Rockwood Experimental Hatchery, Balmoral, Manitoba. Fish were held in 2.3 kL fiberglass tanks supplied with flowing, aerated, dechlorinated Winnipeg city water at 12° C under a 12h L:12h D photoperiod (08:00-20:00). They were fed Ewos (Rundle Feed Mill, Palmerston, Ont.) or Growers trout pellets (Martin Feed Mills, Elmira, Ont.) once daily at a ration of 1-2% body weight. Trout were starved 2-3 days before dissections to decrease bile production. Prior to blood sampling, or other manipulations, trout were anesthetized in a solution (0.07 g/L) of tricaine methanesulfonate (MS 222; Syndel Laboratories Ltd., Vancouver, BC).

2. Protocol for *I infusion

In experiments involving constant infusion of $Na^{125}I$, the $Na^{125}I$ (Na*I) was introduced into 3 trout, ranging in weight from 210 to 260 g, via a surgically-implanted, self-powered Osmotic Mini Pump (model 2001; Alza Corp., Palo Alto, CA.). Pumps were implanted under anesthesia, into the coelom via an incision made ventral and slightly lateral to

the pelvic fins, and the incision was then sutured. At a mean fill volume of 229.0 uL, and at 12° C, the pump continuously released an average of 6.2 µL/day of a Na*I solution into the fish. Concentrations of Na*I used for each fish were 1.0 µCi/µL, 0.5 µCi/µL, and 0.25 µCi/µL. Dilutions were made with deionized and distilled water.

3. Protocol for *T_3 infusion

In experiments involving constant infusion of *T_3, trout of 300-400 g were used. Fish were kept individually in 70-L glass aquaria at a 14h L:10h D photoperiod. Constant infusion of *T_3 was administered by large osmotic pumps (model 2ML1) placed outside of the body. The medium containing *T_3 was a plasma-saline (1:9) solution. Fish were cannulated through the operculum to the dorsal aorta. Detailed cannulation procedures are given in Brown et al. (1986). After cannulation, fish were allowed to recover for 7 days before exposure to acid or acid and aluminum for another 7-day period. Three days after the start of acid or acid and aluminum stress, or after recovery from cannulation for unstressed fish, a single 1.0-µCi priming dose of *T_3 was given to each fish before infusion with 0.05 µCi *T_3/h until isotopic equilibrium (IE) was achieved. IE is defined as the state in which a uniform specific radioactivity has been reached throughout the body when the tracer is administered through continuous uptake (Jacoby & Hickman 1966).

Tissues and plasma samples from eight fish were obtained. Of these, 5 were held at pH 7.7 (unstressed) whereas one of the remaining fish was acid (H_2SO_4)-stressed at pH 4.8, and two were stressed by a combination of acid (pH 4.8) and elevated aluminum concentration (21.6 μM) in the water. Fish were not fed throughout the experiments. All plasma samples were taken at time of death (96 h after *T_3 infusion), and fish were kept frozen (at -22° C) until tis- sues were processed for extraction of T_3. Plasma *T_3 cpm used in determining equilibrium was measured by separation assay using T_3 anti- bodies, and plasma T_3 concentration by RIA.

B. Blood and Tissue Sampling

Fish were anesthetized, blotted dry, weighed (±0.1 g), bled, and killed by concussion. Blood was removed from the caudal vessels with a preheparinized 1-mL turberculin syringe and immediately centrifuged (International Cen- trifuge Model MB) at 15,000 g for 2 min to separate plasma. Plasma was stored in 2-mL plastic vials covered with Parafilm at -20° C.

Whole organs (liver, heart, gastro-intestinal tract, and kidney) and tissue samples (gills, skin, and muscle) were removed from freshly-killed fish. Frozen tissues were

used only from those fish that had undergone constant infusion. A 2-g sample of the tissue was removed unless the organ weight was less than 2 g, in which case the whole organ was removed. Prior to mincing with scissors, tissues were rinsed with a 0.7% saline solution to minimize cell rupture. Rinsing was especially important for heart and intestine, in order to remove trapped blood or food, waste particles and bile, respectively. Bile and intestinal contents were removed to prevent contamination of tissue by iodothyronine conjugates and extracellular T_3s. When dissecting the liver, the intact gall bladder was removed and discarded to minimize bile contamination of surrounding tissues. The gastro-intestinal tract was divided into stomach and intestine. The pyloric cecae were discarded to minimize interference in the assay caused by their high fat content. Stomach samples included the lower portion of the esophagus to the point of insertion of pyloric cecae. The intestine was cut immediately posterior to the pyloric cecae and around the anus. Kidney tissue was scraped off the vertebral column. The heart included the bulbus arteriosus. Gill filaments were cut from intact gill arches. Muscle tissue was removed from the area immediately posterior to the head above the lateral line and includes both red and white muscle. Skin was taken from the area overlying the muscle section.

C. Tissue Breakdown

Both homogenization and enzymatic digestion were examined. Tissues were homogenized with a Micro-homogenizer attachment of a Sorvall Omnimixer (Ivan Sorvall Inc., Norwalk, CT), at 50,000 rpm for 2-5 mins. For tissue digestion, four enzymes were examined; collagenase, trypsin, protease (Sigma Chemical Co.) and pronase (B-grade, Cal Biochem-Behring Corp.). The minced tissues were placed in glass test tubes (16 x 100 mm). Three different amounts of enzymes (0.001, 0.0025, 0.005 g) were dissolved in 3 mL phosphate buffer ($Na_2HPO_4.7H_2O$, 28.6g/L, pH 7.5), mixed with the tissue samples, and placed into a water bath at 37° C for a minimum of 12 h. The buffer also contained 1 mM PTU (propylthiouracil, Sigma Chemical Co.) to prevent possible deiodination of T_3. The efficiency of digestion was visually determined. If tissues broke up easily by stirring, digestion was considered complete. Out of the four enzymes initially chosen, two were proven to be particularly useful. In all subsequent experiments, 0.0025 g collagenase was used for muscle and skin tissues (which contain a high proportion of collagen fibers) and 0.0025 g pronase for the remaining tissues.

D. T_3 Extraction

1. Method I

Pronase and collagenase were dissolved in 0.05M TRIS buffer (pH 7.5) with 1 mM PTU. After tissue digestion was complete, T_3 was extracted by adding 5 mL of ethanolic ammonia (99:1, vol/vol) with 1 mM PTU to the digestion tube. The mixture was stirred and then spun at 3546 rpm (1420 g) for 10 min at 4° C in a Clinicool benchtop centrifuge (Damon/IEC Division). The low temperature helped to precipitate proteins and reduced enzymatic activity. The supernatant was then pipetted off. The addition of ethanolic ammonia followed by centrifugation was repeated twice. All 3 supernatants were pooled and evaporated to dryness in a water bath (37° C) under a fume hood with continuous air flow over each tube. The extract was reconstituted with 1 mL of 0.1N NaOH. When extracts were not immediately assayed, tubes were sealed with Parafilm and kept refrigerated at 4-5° C.

2. Method II

Tissues were digested using phosphate-buffered ($Na_2HPO_4.7H_2O$, 26.8g/L, pH 7.5) enzymes and 1 mM PTU, instead of TRIS buffer. Digested tissues were extracted by ethanolic ammonia (with PTU) and centrifuged. This was repeated twice more. Of the pooled supernatant volume, 3-5 mL were evaporated off under a fume hood, the remainder put

into a 60-mL separatory funnel with 45 mL of chloroform (approximately 3 times the volume of extract) and briefly shaken to mix. (It may be possible to eliminate the step of evaporation to reduce the volume of extract if larger separatory funnels are used). All procedures involving chloroform, which was used to remove lipids, were done under a fume hood. A volume of 15 mL of 2N NH_4OH was then added and mechanically shaken for 5 min to draw off the T_3. After agitation, the contents of the separatory funnels were allowed to sit for 1 h to allow layering and settling of components. This resulted in a clear and frequently pigmented top layer and a cloudy bottom layer. The large bottom layer represented the 'chloroform' layer while the NH_4OH containing the extracted T_3, remained on top. The 'chloroform' layer was drained off and discarded. The 'NH_4OH' layer was drained into test tubes and evaporated under a fume hood in a water bath at 37° C. This final extract was resuspended in 1 mL 0.1N NaOH.

E. T_3 Measurement

1. Direct Measurement of T_3 by Radioimmunoassay (RIA)

Levels of T_3 in tissue extracts or unextracted plasma were measured by an RIA method modified from the one developed by Brown and Eales (1977). Assays were done on 5-mL

columns containing 0.3 g Sephadex G-25 (Quik-Sep, Isolab Inc., Ohio) equilibriated in 0.1N NaOH. The T_3 antibodies (T_3AB; Calchemical Lab Supplies Ltd., Calgary) were approximately diluted with a phosphate buffer (0.1M $Na_2HPO_4.7H_2O$, 0.03M EDTA disodium salt, Fisher Scientific Co., pH 7.4) in order to achieve 50% binding capability at zero concentration of added T_3. This phosphate buffer also served as an eluant in the assay. Working standards of 0-2000 ng% (ng/100 mL) T_3 were prepared by diluting an evaporated stock solution (20,000 ng%) with buffer (KH_2PO_4, 1.198 g; $Na_2HPO_4.7H_2O$, 8.144 g; NaN_3, 0.065 g (Fisher Scientific Co.); bovine serum albumin (Sigma Chemical Co.), 1.00 g/L distilled and deionized water, pH 7.4). *T_3 (Amersham, specific activity of 750 µCi/ug) was diluted with 0.1N NaOH to give a solution providing 5-7000 counts per minute (cpm) in a 100-µL aliquot.

The assay procedure was as follows:

(1) Columns were drained to waste and the bottoms recapped.

(2) A volume of 100 µL of each T_3 standard (0, 31, 62, 125, 250, 500, 1000, 2000 ng%), tissue extracts or plasma were put on columns. Standards were analyzed in triplicate and samples in duplicate.

(3) For each assay, two columns were reserved for non-specific binding (NSB). To these, 0.1 mL of 0.1N NaOH was added in lieu of standard or sample.

(4) A 100-µL aliquot of prepared *T_3 was added directly to each column.

(5) A 100-µL aliquot of prepared *T_3 was also added to 3 separate counting tubes for determination of added dose, i.e. total counts reference (TCR).

(6) Columns were then swirled and drained to waste.

(7) To determine radioiodide contamination of the *T_3, 2 mL of phosphate buffer was put on each colunm and the eluants (containing the iodide and proteins) were collected in this void volume from 3 randomly-selected columns. Uncollected eluants were drained to waste.

(8) Counting tubes were placed under each column and 0.5 mL of T_3 AB solution was added to all columns except the two columns designated as NSB. To these, 0.5 mL phosphate buffer was added.

(9) Columns were covered and incubated at room temperature for a minimum of 12 hours.

(10) After incubation, 2 mL of phosphate buffer was added to elute the antibody-bound *T_3. This results in volumes of 2.5 mL in the counting tubes.

(11) All counting tubes (including TCR) were adjusted to a common 3-mL volume with additional phosphate buffer.

(12) Tubes were sealed with Parafilm and counted to 2% error in a Beckman 5000 Gamma counter at a setting appropriate for ^{125}I isotope window.