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

by

Pearl Y. Fok

A thesis
presented to the University of Manitoba
in partial fulfilment of the
requirements for the degree of
Master of Science
in
Department of Zoology, University of Manitoba

Winnipeg, Manitoba

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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.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. J.G. Eales for his advice and support throughout the years of my project. I thank Drs. L. Campbell (Animal Science) and T. Hara (Freshwater Institute and Department of Zoology) for their valuable critisism of the manuscript. My thanks also to all the people in Dr. Eales' lab who provided experimental help and discussions. I thank Scott Brown who provided the fish isotopically equilibriated with *T_3 and plasma T_3 values. Special thanks to my husband Wolfgang for his encouragement and help.

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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.

(13) Columns were regenerated by sequential elution with an 8-mL solution of human plasma and phosphate buffer (1:10 dilution), 16 mL distilled and deionized water and 4 mL of 0.1N NaOH.

The eluant in counting tubes represents the bound fraction (B). The 'free' (F) $*T_3$ remaining on the column was determined from the difference between B and $*T_3$ initially added (TCR - radioiodide contamination) to each column. After subtracting non-specific elution (NSB), B/F values were probit transformed;

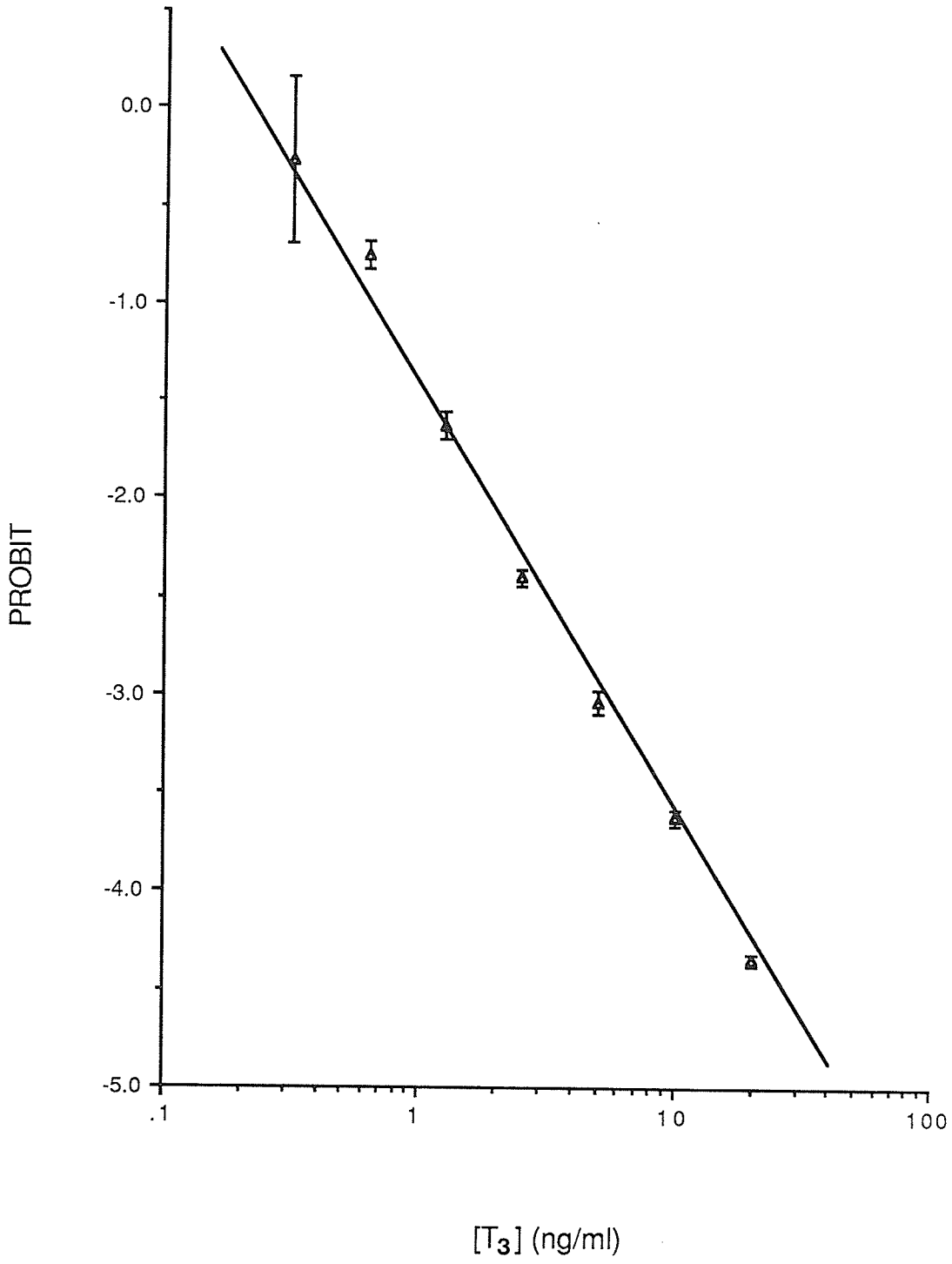
$$\text{probit} = \frac{B/F}{[B/F]_o} \Big/ 1 - \frac{B/F}{[B/F]_o}$$

(where $[B/F]_o$ = bound over free values for the zero standard) and plotted against logarithmic concentration of hormone (Fig. 1). From this standard curve, actual hormone concentrations of tissues and plasma samples were determined by interpolation. All calculations were done using a RIA-data-processing system (Moncayo 1984), employing an Apple IIc computer.

2. Assessment of $*T_3$ in Tissues and Plasma of $*T_3$-Administered Fish

Radioactive tissue extracts were separated to identify $*T_3$ by two chromatographic methods involving use of short or long gel columns.

Figure 1. Typical standard curve for T_3 as determined by RIA. B/F values were probit transformed and plotted against log hormone concentration. Each point represents the mean ($\pm$SE) for 3 replicates. Line is fitted by eye.


0.0
-1.0
-2.0
-3.0
-4.0
-5.0
PROBIT
.1
1
10
100
[T3] (ng/ml)

a) Short-Column Separation

Radioactive T_3 was separated from *I and radioactive iodoprotein (*IP), using a method modified from Eales (1977a). It is based on the principle that specific binding proteins in human plasma will elute TH from the Sephadex column. Assays were done on columns supplied with the Tetralute competetive binding kit, filled with 0.45 g of G-25 Sephadex (fine). Columns were kept stored in 0.1N NaOH at room temperature.

The assay procedure was as follows:

(1) Columns were drained and the bottoms recapped.

(2) Three test tubes per column were labelled for collecting a combination of *I and *IP (one tube) and *T_3 (two tubes).

(3) The first set of tubes was put under columns.

(4) A volume of 100µL of tissue extract was then placed on the column.

(5) A 100 µL aliquot of the injected *T_3 was then put onto 2 columns to determine the *I contamination of the administered *T_3. The amount of radioactivity in tbe sample and put onto the columns did not exceed 10,000 cpm.

(6) Three total counts reference (TCR) tubes were also made by adding 100 µL of the injected *T_3 into the tubes.

(7) Three mL of phosphate buffer (0.1M $Na_2HPO_4.7H_2O$, 0.03M EDTA disodium salt, pH 7.4, Fisher Scientific Co.) were

added to each column, and the *I and *IP eluted. The *T_3 remained bound to the Sephadex.

(8) The second set of tubes was placed under the columns and a 4-mL solution of human plasma and phosphate buffer (1:10 dilution) was added to the columns.

(9) When the phosphate buffer-plasma solution had drained through, the third set of tubes was positioned under the columns and another 4 mL of phosphate buffer-plasma solution was put on the column. The second and third eluents combined, comprised the *T_3 fraction.

(10) All TCR tubes and tubes containing the *I and *IP were made up to a 4-mL volume with additional phosphate buffer.

(11) All tubes were sealed with Parafilm and counted to 2% error in a Beckman 5000 gamma counter.

(12) Columns were regenerated by sequential washing with 4 mL human plasma diluted with phosphate buffer (1:10), 12 mL distilled and deionized water and 4 mL of 0.1N NaOH.

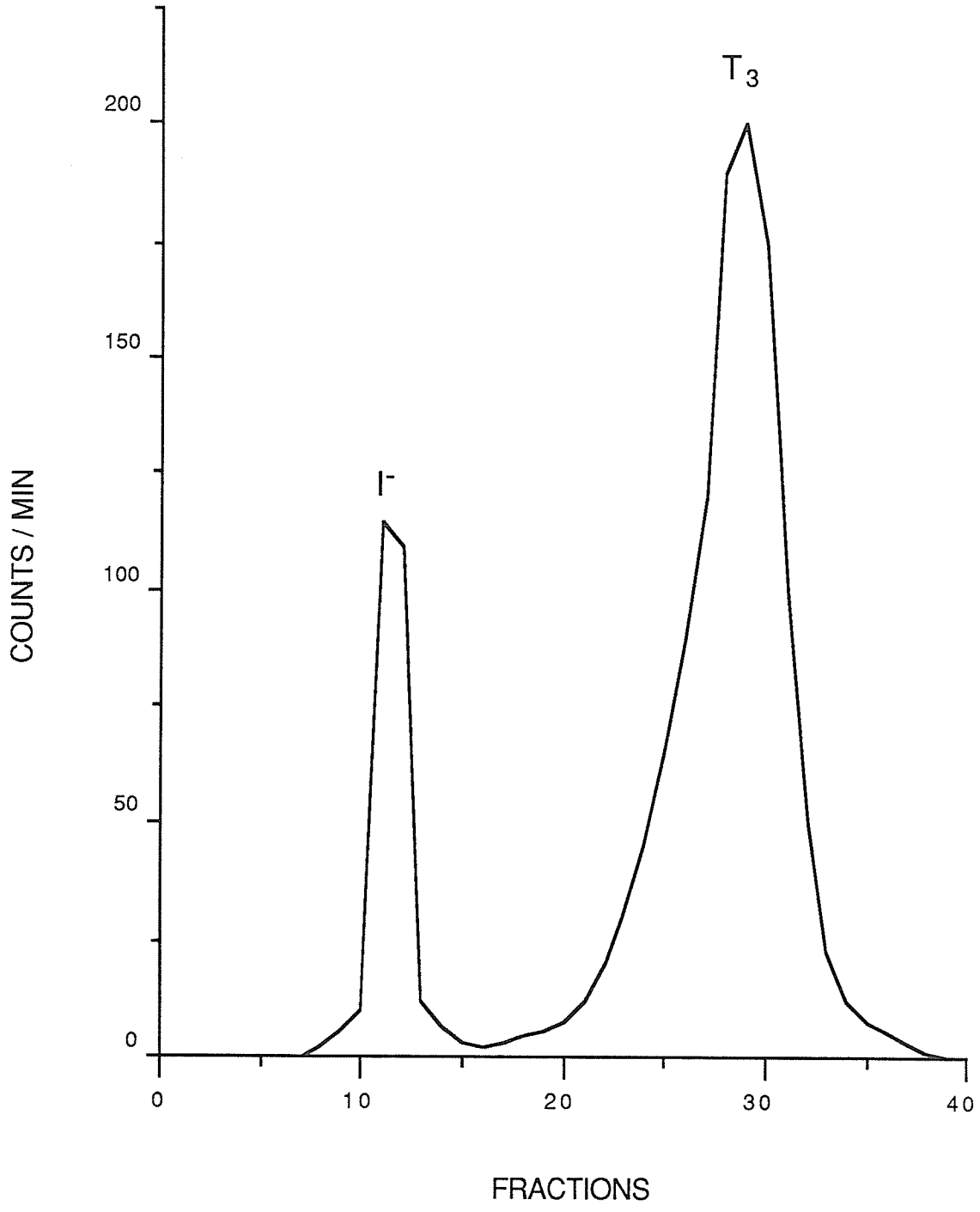
b) Long-Column Separation

Tissue extracts and plasma samples (0.3-0.5 mL aliquots) from constant infusion experiments were put on a long chromatography column (Pharmacia Fine Chemicals Ltd.) of Sephadex G-25 (fine; bed dimensions 27.8 x 1.5 cm). Labelled materials were separated by a 3-part elution system and

collected in 40, 4-mL fractions with an automated system (LKB-Prodkter AB., Bromma, Sweden) consisting of a fraction collector (LKB 2211), HPLC controller (LKB 2152) connected to a peristaltic pump (LKB 2132) delivering 1 mL/min. The column was stored and pre-equilibriated with eluent III (0.1N NaOH; 1.0N NaCl; $Na_2S_2O_8$, 3.6 g/L, Fisher Scientific Co.). Tissue and plasma samples were injected with a 1-mL syringe onto the column, then the tubing was flushed with 3 mL of eluent III. Eluent I (0.1N NaOH; 0.01N NaCl; $Na_2S_2O_8$, 3.6 g/L) removed the first 25 fractions which contained any iodoprotein (fractions 4-5) or iodide (fractions 12-13) present. Eluent II (0.15N NaOH; 0.01N NaCl; $Na_2S_2O_8$, 3.6 g/L) eluted fractions 25 to 37 which contained T_3 (at around fraction 30). Between fractions 38-40, a gradient was set between eluent II and III, which re-equilibriated the column. The collected fractions were counted and a chromatography profile was made. These profiles were compared to a standard profile obtained by chromatographing authentic labelled materials under identical conditions (Fig. 2). Standard elution profiles were made after every 4-5 sample elutions in order to check for any drifting of standard peaks due to changes in column height or other artifacts.

Chromatography of plasma samples after constant infusion of Na*I followed the above procedure with modifications to detect $*T_4$. The total number of fraction collected

Figure 2. Elution profile of ^{125}I-labelled materials of authentic ^{125}I-T_3 as separated by gel filtration. (G-25 Sephadex bed dimensions = 27.8 x 1.5 cm).


T3
200
150
I-
100
COUNTS / MIN
50
0
0
10
20
30
40
FRACTIONS

was 75 (4-mL volume), as T_4 appears approximately at fraction 60. Eluent I was run through the column for fractions 1-25 and 25 to 70 for eluent II. Between fractions 70 and 72, a gradient was set to re-equilibriate the system to eluent III.

The following standardized procedure was adopted in order to objectively quantify the elution profile of radioactive substances for any given tissue under any given condition (i.e. stressed or unstressed):

(1) A chromatograph was selected at random and any fractions with 5 or less cpm were omitted.

(2) The cpm for each fish for that tissue and condition were totalled.

(3) Each total cpm was standardized to a chromatograph from one fish by dividing the one chosen total by each individual total. The chosen standard then equalled 1.0 and the other fractions became correction factors.

(4) The cpm of each fraction was then multiplied by the corresponding correction factor for that particular fish.

(5) The corrected fraction cpm were then plotted for each tissue of each fish.

(6) Mean chromatographs were composed of the mean cpm of each fraction of a particular tissue under a given condition.

By using tissue chromatographs from individual fish, the proportion of the profile that comprised *T_3 (which reflects the proportion of *T_3 in the tissue extract) was determined by the following steps:

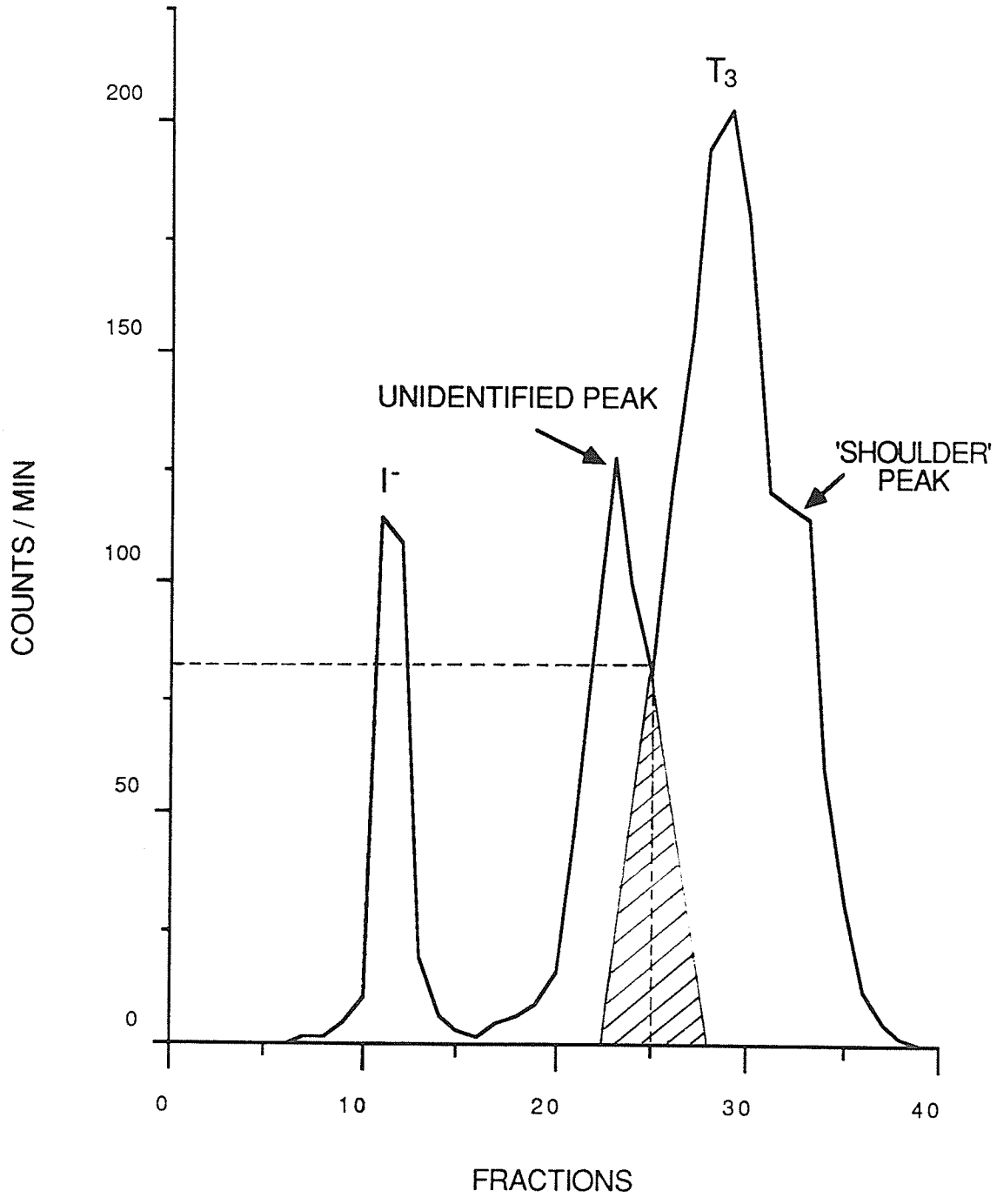
(1) The cpm of all fractions 1–40 were totalled.

(2) The range of fractions that constitute the T_3 peak was selected and the respective cpm were summed. In some elution profiles, no distinct T_3 peak could be detected, usually due to low extract radioactivity in which 'background' counts masked any low T_3 peak. These indistinct profiles were not analysed further.

(3) In instances where the *T_3 peak tended to merge with adjoining peaks, the fraction that fell at the lowest point between the two peaks was determined. The cpm value on the Y-axis corresponding to this lowest fraction was halved and added to the T_3 fraction, thereby accounting for the overlap of areas between adjoining peaks (Fig. 3). This method was applied only when a definite trough existed between peaks, and not when a major *T_3 peak developed a 'shoulder' (Fig. 3). In most cases, the 'shoulder' was small and its radio activity included in the *T_3 fraction.

(4) The radioactivity representing the *T_3 peak was divided by the total radioactivity (total cpm) from all fractions, providing the proportion of total tissue radio-

Figure 3. Example of determining cpm values for T_s fraction from an elution profile with merging peaks. Overlapping areas of the peaks are shaded.


200
150
100
50
0
COUNTS / MIN
T3
UNIDENTIFIED PEAK
I-
'SHOULDER' PEAK
0
10
20
30
40
FRACTIONS

activity existing as *T_3.

(5) This proportion was then multiplied by the total radio activity in the original 1-mL extract reconstituted in 0.1N NaOH.

(6) The resulting cpm was corrected for isotopic decay (t1/2 of ^{125}I = 60 days) to the time of death and was used in the equation for the calculation of specific activity.

3. Use of the Constant Infusion Principle to Determine Tissue T_3 Content.

In trout that have been constantly infused with *T_3 to the point where the plasma *T_3 has risen to a plateau, it is assumed that a steady state (isotopic equilibrium) exists with regard to *T_3 exchange between the various physiological compartments of the fish. In such a steady state the *T_3 specific activity (SA = cpm *T_3/ng T_3) is equal throughout the fish. To obtain the SA value, the T_3 cpm and the T_3 concentration were determined for the readily-measured plasma samples, and the appropriate corrections for radioactive decay applied. Actual plasma values used to calculate SA are given in Appendix 1.

An attempt was also made to reach isotopic equilibrium by constantly infusing trout with Na*I. In this case, endogenous iodothyronine formation from the *I would be detected

as *T_3 and *T_4. The SA value would be calculated from the Na*I and iodide levels in the plasma.

F. Estimatiion of T_3 Content in Whole Organs

The T_3 content of tissues and whole organs of a hypothetical 300-g fish were determined by multiplying the T_3 concentration (T_3 mass per gram tissue) by the organ weights calculated from regression equations.

Regression equations relating body weight to organ weights were determined from body and organ weights of 48 trout. Wet weights ranged from 171-702 g, with 36 of the fish over 300 g in weight. Since only 2-g aliquots were taken from muscle and skin, no equations could be calculated for those two tissues.

G. Statistical Analyses

Statistical analyses were done using the Statistical Analysis System (SAS Inst. Inc. 1982). Differences between experimental groups were tested by one-way analysis of variance (ANOVA). If an ANOVA test revealed that group means were significantly different, Duncan's new multiple range test was used to test differences between tissues. A probability level of ≤ 0.05 was considered significant. The relationship between organ weights and body wet weights was determined by the least-squares linear regression.

RESULTS

A. Establishment and Validation of Procedures

This section deals with the validation of methods I and II for digestion and extraction of T_3 from tissues. While method II was the final method chosen, the validation of method I, and its limitations, are presented since it is a modified method successfully used by other researchers (Gordon & Spira 1975; Kobuke et al. 1987), and may be of value in studies of non-fatty tissues. Some of the materials and methods used in these preliminary experiments are included in the 'Results' section to simplify the previous 'Materials & Methods' section which has been restricted to the general and validated procedures.

1. Enzymatic Digestion and Homogenization

The enzymatic digestion and homogenization procedures were compared on livers and kidneys from fish injected intraperitoneally 12 h previously with 1.5 µCi $*T_3$. Equal parts of each tissue were either digested by pronase or homogenized. $*T_3$ was recovered by 6 successive alcohol extractions (method I). The total amount of $*T_3$ recovered from all 6 extractions, as determined by short-column assay, was similar for the two methods of tissue breakdown, and in all cases over two-thirds of this total was recovered in the

first 3 supernatant extracts (Fig. 4). However, enzymatic digestion provided a higher percent recovery of *T_3 in the first three supernatants and this difference was greater for liver (89.9% versus 79.9% for digestion and homogenization, respectively) than for kidney (70.7% versus 68.9%). It was also found that the residual pellet radioactivity (which may include labelled material other than *T_3) was higher in homogenized than digested samples. Attempts to homogenize other tissues (e.g. heart, skin, and stomach) did not result in adequate tissue breakdown. A further advantage of enzymatic digestion was that it allowed simultaneous processing of numerous tissue samples, without the danger of cross contamination and the necessity of laborious cleaning of the homogenizer containers, and blades. For these reasons, it was decided to use enzymes for tissue breakdown and to use only the first 3 supernatants for extraction.

Enzymatic digestion may be extended to T_3 itself. To test this, *T_3 was added to the digestion tube (containing enzyme dissolved in 3 mL TRIS buffer and 1 mM PTU) before or after incubation, but in the absence of any tissue. The amount of *T_3 present after incubation (12h at 37° C) was determined after extraction and short-column separation. No differences in the absolute or relative amounts of radioactivity appearing in the *T_3, *I, and *IP fractions before and after enzyme digestion could be detected.

Figure 4. Recovery of *T_3 from liver (A) and kidney (B) after homogenization or enzyme digestion. Six successive supernatant washes were examined. Bars represent means (±SE) of 3 fish. (See App.2 for actual values).

2. Extraction Efficiency of Method I

a) Effect of Digestion Enzymes Alone on *T_3 Recovery

To determine the efficiency of method I in recovering *T_3 in the presence of enzymes but in the absence of tissue, a series of tubes containing the following solutions was set up in triplicate: (1) *T_3 + TRIS buffer (2) *T_3 + TRIS + collagenase (3) *T_3 + TRIS + pronase. A volume of 3 mL of buffer with 1 mM PTU was used to dissolve 0.0025 g of enzyme. The absence of tissue controlled for variability in recovery caused by possible binding of T_3 to tissue proteins. Recovery of total added radioactivity (43,000 cpm) ranged from 80.5-98.0% . The presence of the enzyme did not effect recovery.

b) Effect of Buffer on T_3 Recovery

The procedure was repeated in an attempt to recover 4 ng of T_3. An RIA was performed on the final extract. The added T_3 recovered ranged from 71-135% . Since extraction method I was shown above not to interfere with the recovery of *T_3, and since the wide range in recovery of unlabelled T_3 was similar with either enzyme used, it was assumed that the inconsistent unlabelled T_3 recovery resulted from RIA interference by the TRIS buffer. Consequently, a phosphate buffer ($Na_2HPO_4.7H_2O$, 26.81 g/L, pH 7.4 + 1 mM PTU) was tried as an enzyme solvent. Both *T_3 (9,000 cpm) and

unlabelled T_3 (4 ng) were again added separately. Recovery of unlabelled T_3, as determined by RIA, ranged from 95 to 119%, whereas recovery of added $*T_3$ ranged from 90-99% . In all subsequent experiments, phosphate buffer was used to dissolve enzymes.

c) Interference by Lipids and Other Materials

A major problem in this extraction procedure was the accumulation of particulate and/or lipid-like material in resuspended tissue extracts. Both materials tended to block pipettes or clog Sephadex columns. To alleviate these problems, aliquots of the final 1 mL of extract were centrifuged in conical vials at 15,000 g for 3 min at room temperature. This caused layering of the extract. If present, the lipid layer sat on the top, followed by a clear center portion and occasionally, particulate material on the bottom. Tissues with the most viscous extracts and the most undissolved particles came from liver, kidney, gill, and heart.

T_3 may be lost by either trapping in the lipid layer, which was discarded (since thyroid hormones are known to be lipid soluble; Hillier 1970) or binding to particles in the bottom layer. To examine the above possibilities, 20 µL of $*T_3$ (1800 cpm) was mixed with liver, kidney, and gill samples before enzyme digestion, and then processesed by

method I. From the final reconstituted tissue extracts, 100 µL was removed and counted in 1 mL NaOH. This represented the unspun sample. The remaining tissue extract was spun down and 100 µL of the clear supernatant was removed and also counted. The radioactivity recovered from unspun samples was 76% for kidney, 58% for liver, 100% for heart and 71% for gill, and for spun samples, was 63% for kidney 37% for liver, 100% for heart, and 37% for gill. Loss of radioactivity before and after centrifugation varied, depending on tissue type. In addition to the partial loss of T_3, removal of either lipid or particulate matter resulted in a very small volume of extract remaining available for analysis. Whenever an aliquot of extract was removed, the remaining sample also became less and less homogeneous.

There was evidence also that other substances that might interfere with the RIA were extracted with the T_3. Extracts from liver, kidney, gill and heart were serially diluted (100, 50, 25, 12.5%) and T_3 measured by RIA. Larger than expected amounts of T_3 were detected following dilution, indicating the possibility of some interference with the binding of $*T_3$ to the T_3 antibody for these tissues.

d) Review of Method I

In reviewing method I up to this point, it seems that (1) enzymatic digestion does not quantitatively or qualitatively affect the T_3 content of the tissue, (2) TRIS buffer should be replaced by phosphate buffer as the former interferes with the assay, (3) collection of 3 supernatants is sufficient to recover over two-thirds of the T_3 after extraction with ethanolic ammonia, and (4) no satisfactory method had been found yet for removing lipids from tissue extracts. These findings suggest that method I is only reliable for non-fatty tissues and therefore inadequate for this particular study. The remaining sections deal only with method II.

3. Extraction Efficiency of Method II

a) Extraction of *T_3

1) Effect of Extractants

Two extractants were tested in separate experiments. Acidified alcohol (5 mL ethanol with 1 mM PTU, 0.4 mL 2N HCl), as previously used by Nejad et al. (1975) and van Hardeveld and Kassenaar (1976), did not work well. In both the presence and absence of tissues, white particles formed in the final extract. When the final reconstituted extract was centrifuged (15,000 g, 3 min at room temperature) to

remove the particles, approximately 50% of added radioactivity was lost from the sample when compared to unspun samples. Use of ethanolic ammonia resulted in a reduced particulate formation in the final reconstituted extract, thereby avoiding centrifugation.

2) Effect of Digestion Alone on *T_S Recovery

To test the extraction efficiency of method II on a *T_S source and enzyme alone, the extraction was carried out in the absence of tissue. This would reveal if the enzymes or buffer used to dissolve the enzymes has any affect on *T_S recovery when extraction is carried through a chloroform-NH4OH wash. *T_S (7,000 cpm) was added to 1) phosphate buffer, 2) phosphate buffer and collagenase, and 3) phosphate buffer and pronase. In addition, a standard (tube 4) contained *T_S and buffer and was not run through the extraction procedure.

To determine if the NH_4OH efficiently withdrew T_S from the chloroform-ethanol extract, 1-mL aliquots of each layer were taken after mixing in the separatory funnels, counted, and multiplied by their respective total volume of each fraction. When actual tissue extracts were used, the volumes of chloroform and NH_4OH layers varied due to lipid compositions of the tissue extracts, i.e. for some tissues a larger or smaller NH_4OH layer appeared after mixing.

For conditions 2 and 3, containing the enzymes, the 1-mL chloroform aliquot had 0 cpm. The chloroform layer of sample 1 (phosphate buffer only) contained 4% of the counts of the standard. The cpm from the NH_4OH layer represented 89%, 87%, and 88% of those of the standard 4, for samples 1, 2, and 3 respectively. The NH_4OH layer not only contains most of the hormone, but the enzymes do not affect this extraction procedure.

3) Homogeneity of T_3 in Lipid Reduced Extracts

The problems caused by fatty, viscous tissue extracts were also greatly reduced by the use of chloroform and NH_4OH. Muscle and skin, which caused problems in method I, now contained the least amount of particulate material in the final extract of method II. The particulate matter that did appear, was allowed to settle out and the extract samples were removed directly from the test tube as needed. Tissues to which $*T_3$ was added, were extracted and 100-µL aliquots were counted. These 100-µL samples contained 10% of the total cpm of the final 1-mL extract, indicating that the 1-mL extract had homogeneous T_3 distribution and was not binding preferentially to the material that settled out. Short-column separation demonstrated normal recovery patterns with all radioactivity accounted for in the eluents when compared to a standard containing the total added radioactivity.

4) Recovery of *in vivo* and *in vitro* Introduced *T_3

To determine how well extraction method II recovered T_3 from tissues, a *T_3 source was introduced into the tissues both *in vitro* and *in vivo*. For recovery *in vitro*, *T_3 was added to the tissue before digestion (i.e. to the earliest step of the extraction procedure). The added *T_3 served as a standard and represented 100% recovery. Mean percent recovery ranged from 63.5% for muscle to 74.0% for intestine, and did not differ statistically between tissues (Table 1). The *in vitro* method required *T_3 to be added to tissue already removed from the fish, and therefore no longer metabolically representative of living tissue. A more realistic approach is to test *T_3 recovery from a fish that has been allowed to take *T_3 into its own tissues. Following *T_3 administration *in vivo* using a cannula in the dorsal aorta, it was shown that mean percent recovery (cpm of the digested tissue constituted 100% recovery) ranged from 55.8% for kidney to 83.8% for heart (Table 1), and differed significantly between tissues (ANOVA; $p \leq 0.005$). Recovery seemed to vary more between tissues *in vivo*. Since the *in vivo* addition reflected the natural situation more accurately, in all following experiments, the amount of T_3 recovered in each tissue was corrected for the extraction efficiency found in *in vivo* to determine total (theoretical) amounts of T_3 present in the tissues.

Table 1. Radioactivity recovered from trout tissues after *in vitro* and *in vivo* addition of $*T_3$. Values represent mean (±SE) percent recovery of 3 fish after extraction. (See App.3 for individual values).

Tissue	% recovery		correction factor[a]
	in vitro	*in vivo*	
Liver	67.2 ±0.4[b,c]	75.2 ±0.9[d]	1.33
Kidney	67.4 ±5.0	55.8 ±1.7	1.80
Gill	69.5 ±6.3	82.8 ±2.8	1.21
Heart	72.7 ±6.7	83.8 ±6.0	1.19
Stomach	72.3 ±4.9	80.8 ±6.4	1.24
Intestine	74.0 ±6.4	82.2 ±6.2	1.22
Muscle	63.5 ±1.1	68.3 ±2.4	1.46
Skin	73.0 ±2.7	72.6 ±3.8	1.38

[a](100/x), calculated from *in vivo* data
[b]added dose CPM =100%
[c]n=2
[d]digested tissue CPM =100%

Plasma samples did not undergo any extraction or correction procedures since they were placed directly onto Sephadex columns.

b) Recovery of Unlabelled T_3

RIA has been successfully used for the measurement of thyroid hormones in trout plasma (Brown & Eales 1977). Before the same RIA could be used routinely for measuring T_3 in tissue extracts, a number of possible sources of error had to be investigated: (i) How reproducible is the RIA method used in this study? (ii) Is the substance determined by RIA actually T_3? (iii) Does the RIA reliably determine T_3 concentrations from the tissue extract? i.e. is the extraction procedure compatible with the RIA?

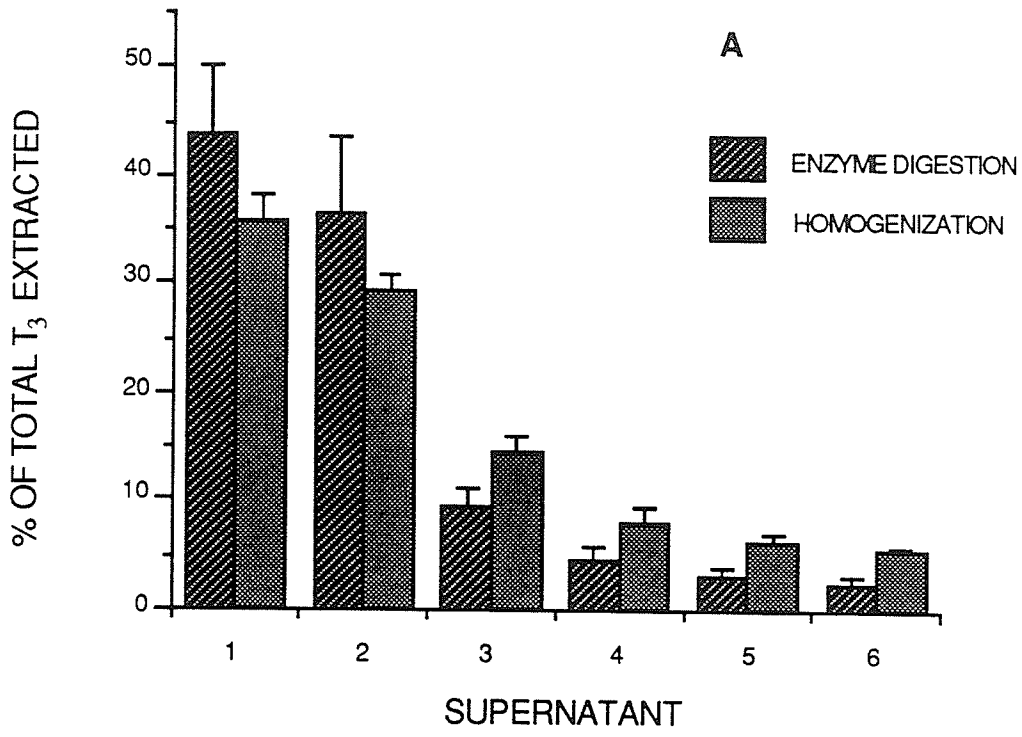
(i) Coefficient of variation (CV%) was used to determine 'within-' and 'between-' assay reproducibility using a 10 µL standard. For 6 RIAs, the 'within-'assay CV% were 4.8%, 3.7%, 2.9%, 3.8%, 5.2%, 8.4% . The CV% between the 6 assays was 9.4% .

(ii) To substantiate that the tissue extracts contained measurable T_3, extracts were serially diluted with 0.1N NaOH to 50%, 25%, or 12.5% of the original concentration and T_3 concentrations were compared to those expected from the theoretical dilution pattern. If T_3 is the only ligand bind-

ing to the T_3 antibody, the T_3 concentration will follow the expected dilution in relation to the standard curve generated in an RIA. If other ligands are being measured with different binding kinetics to the T_3 antibody, the dilution profile will differ from that of the standard curve.

To compare the T_3 concentration of diluted samples to the dilution pattern, the 100% (undiluted) sample was assumed to represent the actual concentration determined by RIA. The T_3 concentration of each diluted sample was then calculated as a percentage of the value of the undiluted sample. The T_3 content of all tissues closely followed the expected concentrations (Fig. 5). This is consistent with results of a typical RIA standard curve, indicating that the substance measured by RIA in the tissue extracts is probably T_3.

(iii) Although there is acceptable recovery of $*T_3$ from tissues, the recovery of unlabelled T_3 from the extract, as measured by RIA, still had to be determined. Since endogenous T_3 concentrations were unknown, a known dose of unlabelled T_3 was added to each tissue and compared to simultaneous estimate of the endogenous T_3 values by RIA. Equal amounts of each tissue from 2-3 fish were pooled, minced by hand, mixed, and divided into 2 portions of equal weight. One portion served as a control and was examined for the


A
ENZYME DIGESTION
HOMOGENIZATION
% OF TOTAL T_3 EXTRACTED
50
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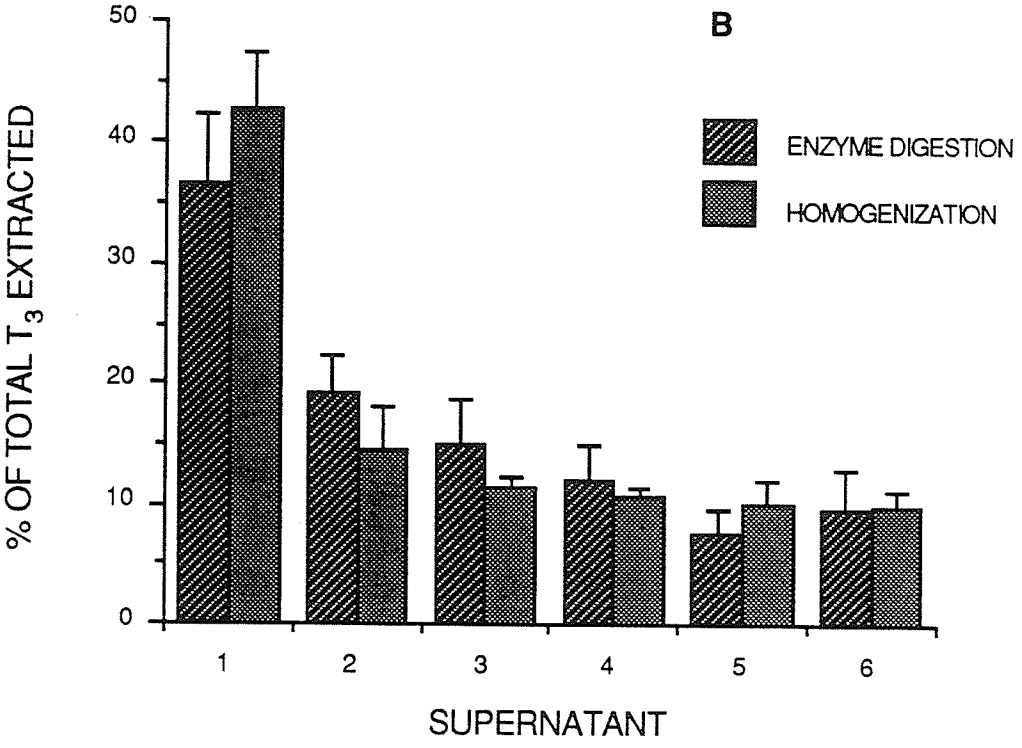
B
ENZYME DIGESTION
HOMOGENIZATION
% OF TOTAL T_3 EXTRACTED
50
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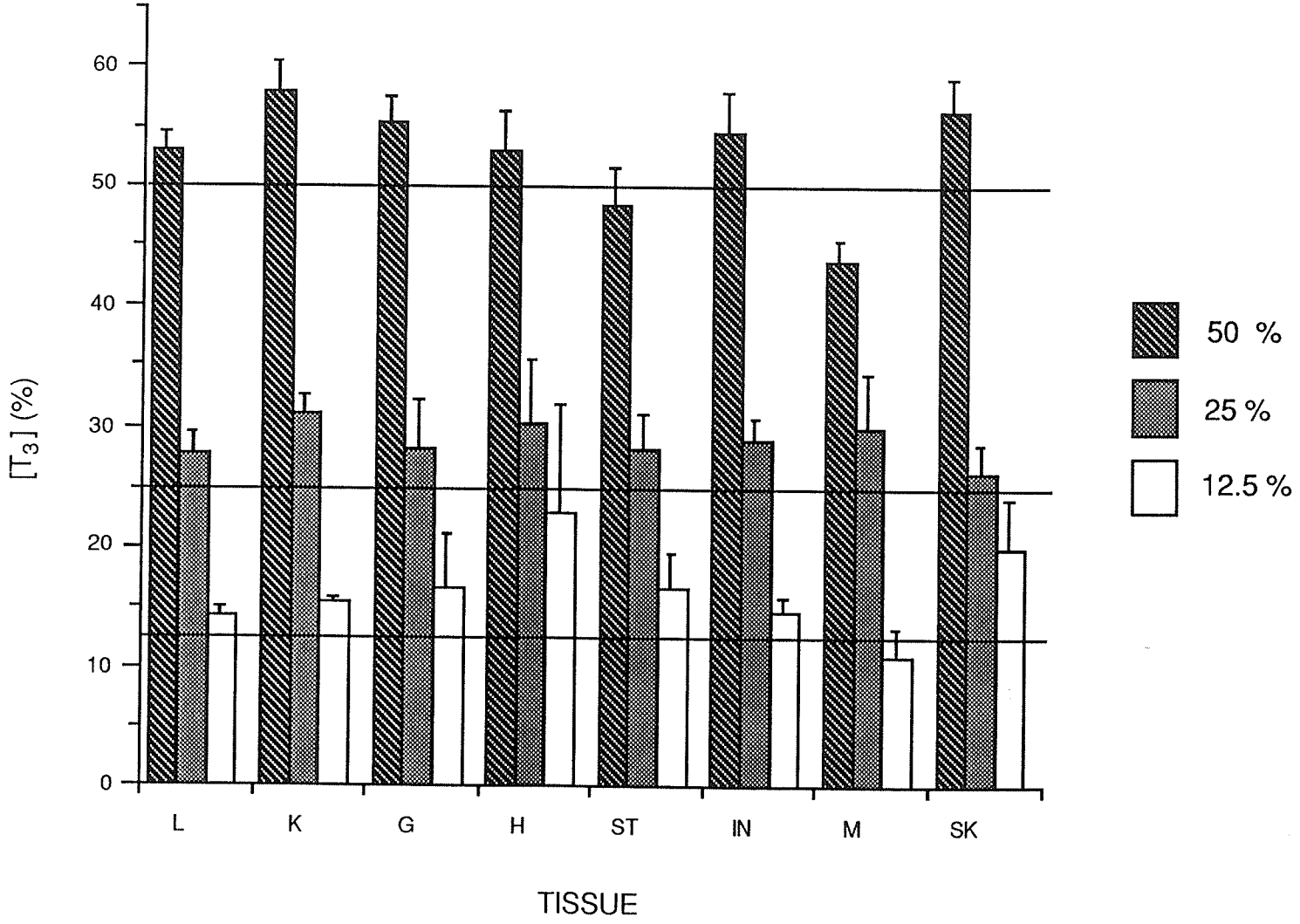
SUPERNATANT

Figure 5. Percent T_s recovered (determined by RIA) after three serial dilutions (50, 25 and 12.5%) of extracts. Bars represent mean (±SE) percentages of 7-9 fish. Horizontal lines indicate expected concentrations. Undiluted extracts (not shown) equal 100%. (See App.4 for actual values). L=liver, K=kidney, G=gill, H=heart, ST=stomach, IN=intestine, M=muscle, SK=skin.


60
50
40
30
20
10
0
[T3] (%)
L
K
G
H
ST
IN
M
SK
TISSUE
50 %
25 %
12.5 %

endogenous T_3 level while 5 ng of unlabelled T_3 were added to the other portion. All tissue samples were digested and T_3 extracted. For almost all tissues, samples with the added unlabelled T_3 had higher concentrations of T_3 than their respective control group. However, in most cases, the amount recovered was only a fraction of the added T_3 dose (Table 2). For one liver and one stomach sample, recovery was actually greater than the added T_3 dose and in three cases (one liver, kidney and intestine) less T_3 was recovered than present in the respective control (Table 2). Overall, percent recovery ranged from 0 (when control T_3 exceeded experimental), to 138.6% (when T_3 recovered exceeded 500 ng%). Recovery of T_3 did not seem to be tissue specific. Blood plasma taken from individual fish contributing tissues to the pooled samples, showed no abnormally high or low T_3 concentrations when analyzed by RIA. The experiment was conducted 3 times (trials 1, 2, and 3) and equally inconsistent recovery was obtained in each case.

In one of the above T_3 recovery trials, 2 standards were set up simultaneously with the pooled tissue samples. Neither standards contained tissue, but in standard 1, 5 ng of T_3 was added to 3 mL phosphate buffer (volume of buffer used in dissolving enzymes in an extraction), then run through the extraction procedure together with the tissue samples. The second standard also contained 5 ng of

Table 2. T_3 (ng%) recovered (as determined by RIA) after addition of 500 ng% T_3 to tissues ($+T_3$) or without added T_3 (control). Values represent means of replicate samples. Trials represent tissue pools of 2-3 fish.

	Trial 1		Trial 2		Trial 3	
Tissue	control	$+T_3$	control	$+T_3$	control	$+T_3$
Liver	623.1	840.9	646.0	580.2	1012.7	1644.4
Kidney	384.9	636.5	375.0	703.4	579.0	391.7
Gill	241.2	493.3	429.1	624.3	351.0	437.1
Heart	-	-	437.8	693.2	289.5	671.1
Stomach	1585.4	2278.4	515.9	720.3	396.7	603.5
Intestine	-	-	576.7	679.2	545.7	378.5
Muscle	132.7	397.3	339.7	520.7	222.1	239.8
Skin	291.2	567.8	476.5	741.2	425.8	121.5

unlabelled T_3 in 1 mL of 0.1N NaOH which equalled the final volume of a reconstituted extract sample. The second standard was put onto RIA Sephadex columns to determine the added amount of T_3 directly. Comparison of this value to that obtained from the first standard, would indicate loss of unlabelled T_3 through the extraction procedure alone. If both values showed the same discrepency in the amount recovered by the RIA, it would indicate that this discrepency stems from the RIA and not the extraction procedure itself. Results for both standards showed higher T_3 recovered (688.5 ng% standard 1, 532.2 ng% standard 2) than the added dose of 500 ng% .

It had been assumed that if T_3 concentrations differed between standards, standard 1 should show a lower T_3 concentration than in standard 2 because only the former underwent extraction, with possible loss of some T_3 during that process. This suggests that for standard 2, recovery of T_3 is probably 100%; whereas standard 1 shows that the extraction procedure (and not tissue) may cause the RIA to overestimate the T_3 concentration.

Because of the inconsistencies in RIA-determined T_3 concentrations after the addition of a known amount of unlabelled T_3 to tissue samples, a serial dilution was again performed (50%, 25%, 12.5%) to determine whether the added

T_3 in the tissue extract, like the endogenous T_3, could be recovered according to the applied dilution pattern. T_3 concentrations found after dilution corresponded well to expected concentrations (Fig. 6). The deviating results for heart tissue in the control group (Fig. 6A) are possibly due to a missed 50% dilution. Thus the use of serial dilution does not conclusively indicate that a substance is T_3.

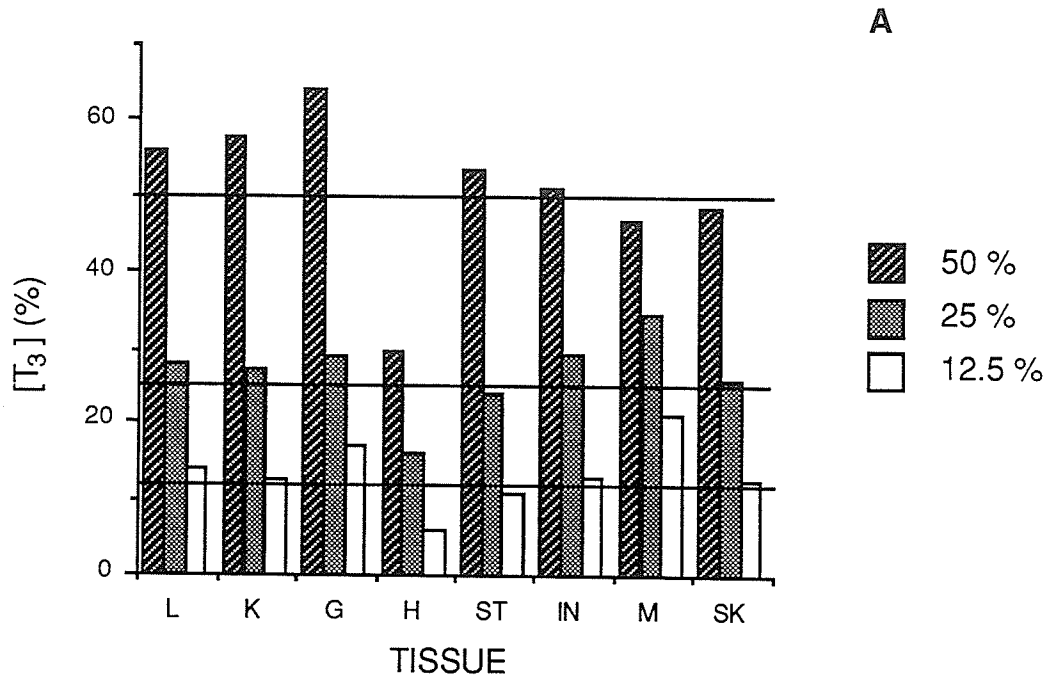
B. Measurement of T_3 Tissue Concentrations

1. T_3 Concentrations Determined by RIA

Despite the shortcomings of the direct RIA determination, tissue T_3 measurements were made by this method for comparison with the IE method.

Four fish ranging in weight from 312.0 - 453.0 g were analysed for tissue T_3 by RIA. Mean T_3 concentration ranged between 1.05 to 3.34 ng/g tissue, and did not differ significantly among tissues (ANOVA, $p>0.1$; Table 3). Kidney and liver values were highest, followed by heart, intestine, and stomach; gill, skin and muscle had lowest concentrations. Tissue/plasma (T/P) ratios of T_3 concentrations were calculated. For all tissues, mean T/P ratios were <1.0 (Table 3). Differences between tissues were not significant ($p=0.06$).

Figure 6. Percent T_3 recovered (determined by RIA) after three serial dilutions (50, 25 and 12.5 %) of extracts without added T_3 (A) and with added T_3 (B). Bars represent [T_3] as percent of undiluted extract. Horizontal lines indicate expected concentrations. For tissue abbreviations, see Fig.5. (See App.4 for actual values)


A
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[T3] (%)
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G
H
ST
IN
M
SK
TISSUE
50 %
25 %
12.5 %

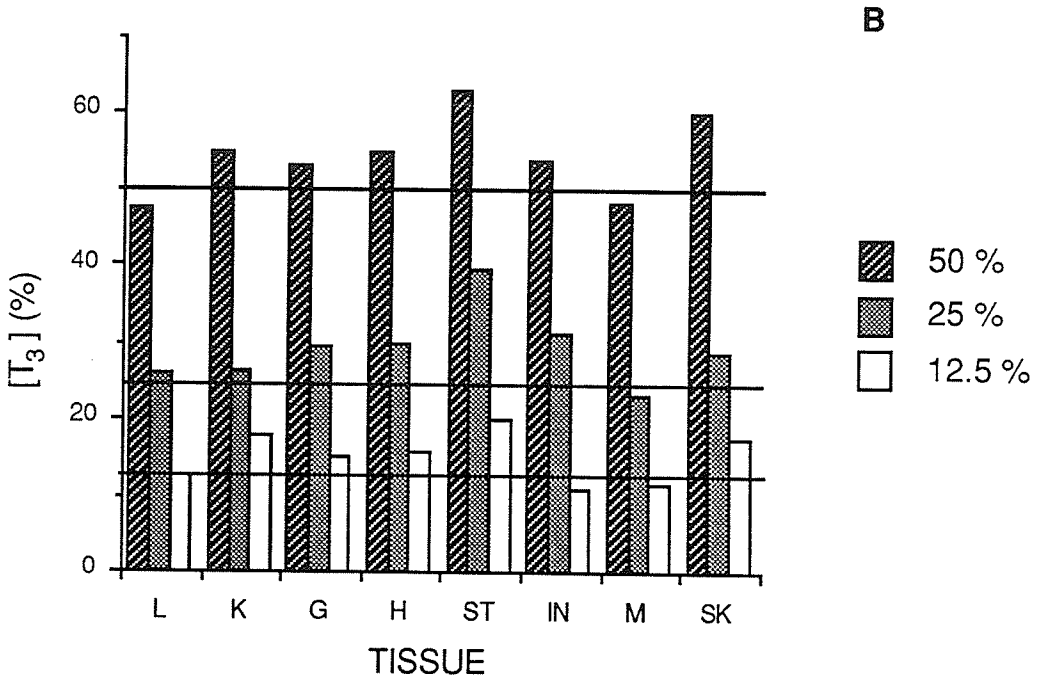

B
60
40
20
0
[T3] (%)
L
K
G
H
ST
IN
M
SK
TISSUE
50 %
25 %
12.5 %

Table 3. Tissue T_3 concentration and ratio of tissue to plasma (T/P) T_3 concentration determined by RIA. Values represent means (±SE) of 4 fish. Data are corrected for 100% extraction. (see App.6 for individual data).

Tissue	T_3 (ng/g tissue)	T/P
Liver	3.09 ±0.64	1.01 ±0.26
Kidney	3.34 ±1.18	0.95 ±0.23
Gill	1.74 ±0.54	0.51 ±0.11
Heart	2.39 ±0.71	0.72 ±0.18
Stomach	1.94 ±0.97	0.47 ±0.05
Intestine	2.24 ±0.46	0.73 ±0.18
Muscle	1.05 ±0.32	0.34 ±0.14
Skin	1.42 ±0.56	0.38 ±0.03
Plasma	3.83 ±1.59	-

T_3 concentrations for pooled tissues, taken from the control group of the T_3 recovery experiment, showed a similar ranking profile, except that heart ranked first (Table 4) and means for uncorrected values differed significantly between tissues.

2. T_3 Concentrations Determined by IE of *I

The objective was to determine the presence and distribution of labelled iodothyronines in plasma and tissues when providing the trout with a constant and continuously supplied source of *I from which labelled hormones might be synthesized. Plasma samples were taken on days 10, 30, and 35. The mean percent increase in cpm/mL plasma for the 3 fish were 15.5% from day 10 to day 20 and 6.2% from day 30 to day 35. Although isotopic equilibrium was not achieved, fish were killed on day 35.

Radiochromatogram profiles from plasma samples showed high *I levels at all time periods but no peaks indicating any thyroid hormone formation. Since no labelled thyroid hormones were detected in the plasma, it was highly unlikely that thyroid hormones would be detected in tissue samples. Only one liver sample was extracted and eluted through the long Sephadex column. No labelled thyroid hormones were detected.

Table 4. Concentration of T_3 (ng/g tissue) for pooled tissues (n=2-3 fish) determined by RIA. Values are corrected for 100% extraction.

Tissue	Trial 1	Trial 2	Trial 3	x ±SE
Liver	4.15	4.30	6.73	5.06 ±0.84
Kidney	3.46	3.38	5.22	4.02 ±0.54
Gill	1.46	2.60	2.24	2.10 ±0.34
Heart	-	6.51	6.89	6.70 ±0.19
Stomach	9.83	3.20	2.46	5.16 ±2.34
Intestine	-	3.51	4.44	3.98 ±0.47
Muscle	0.96	2.48	1.62	1.69 ±0.44
Skin	2.01	3.28	2.94	2.74 ±0.38

3. T_3 Concentration Determined by IE of $*T_3$

$*T_3$ equilibrium was achieved by 72 h post infusion as determined by a levelling of plasma $*T_3$ over time (Fig. 7).

Elution profiles from both standard (Fig. 2) and tissues (Figs. 8–16) showed that iodide was consistently eluted in fractions 12–13 and T_3 in fractions 28–32. Theoretically, monoiodotyrosine (MIT), diiodotyrosine (DIT), or diiodothyronines (T_2), if present in a sample, could be detected as smaller peaks between those of iodide and T_3; reverse T_3 (rT_3) and T_4 should come after the T_3 peak, respectively.

T_3 represented approximately one-third of the total radioactivity on the chromatographs (Table 5). For unstressed fish, mean percentages of T_3 differed significantly ($p=0.0001$), with muscle tissue being higher than all other tissues (Table 5). In stressed fish, percent T_3 in the chromatographs was similar for all tissues.

Elution profiles (Figs. 8–16) showed that the remaining two-thirds of the radioactivity was largely distributed at fraction 11–13, representing a prominent $*I^-$ peak. For all tissues, and plasma, a peak appeared at approximately fraction 5. This peak was prominent for liver, kidney, gill, and particularly plasma. All tissues examined contained

Figure 7. Plasma radioactivity of rainbow trout during infusion with 0.05 µCi *T₃/h. Values represent means (±SE) of 5 fish. This study and data represent unpublished results provided by Scott Brown.

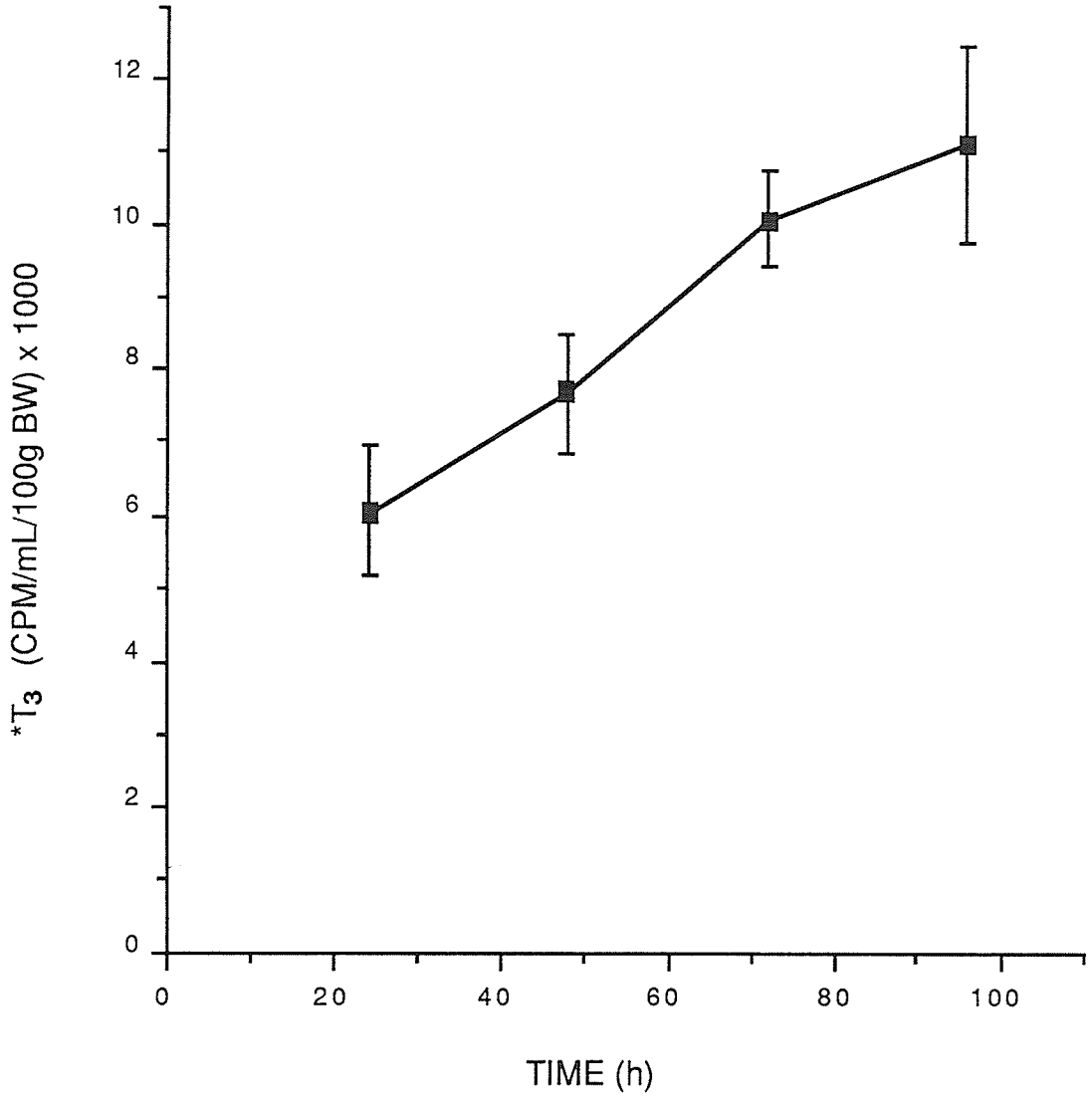
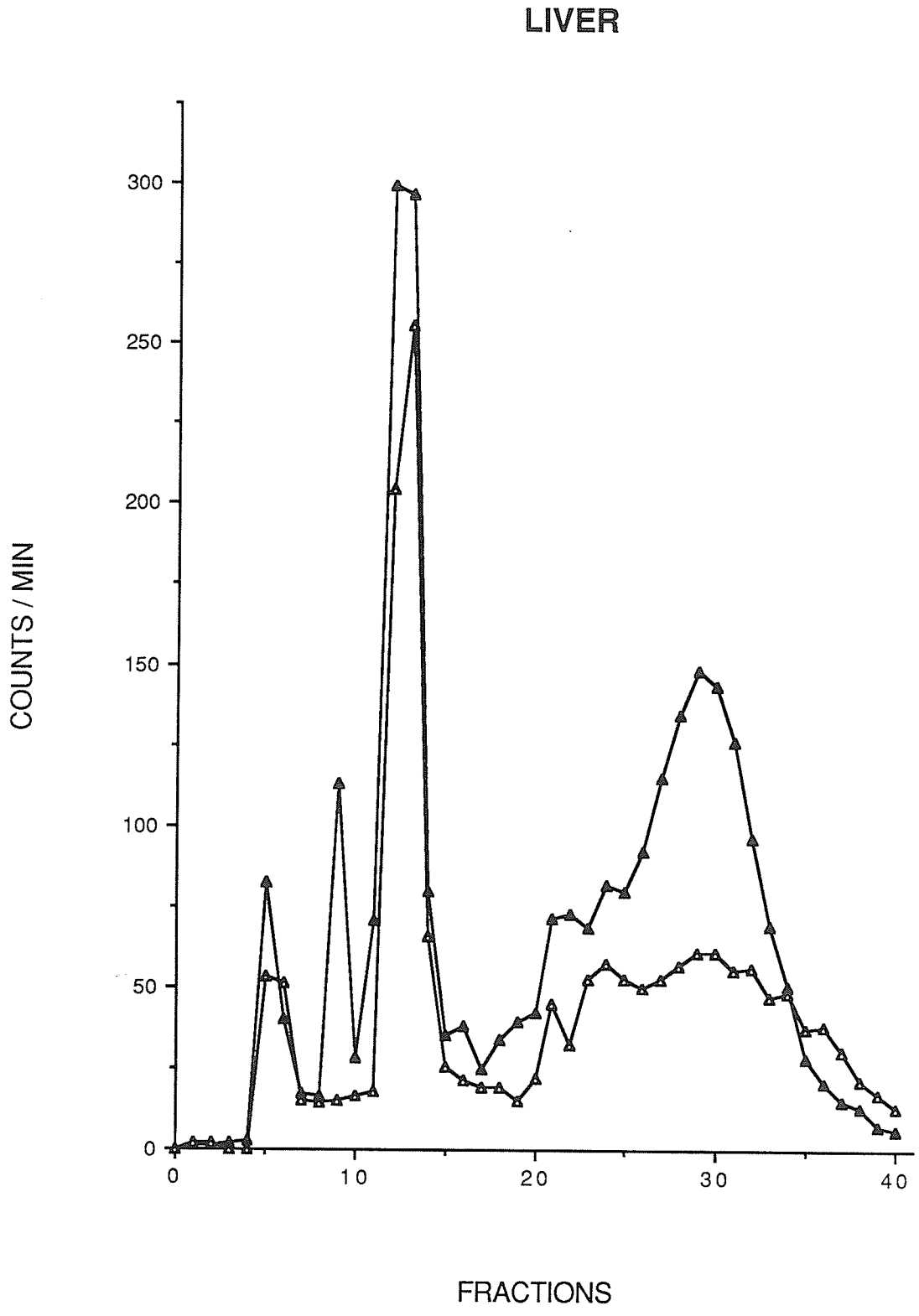

12
10
8
6
4
2
0
0
20
40
60
80
100
*T3 (CPM/mL/100g BW) x 1000
TIME (h)

Figure 8-16. Elution profiles of ^{125}I-labelled materials of tissue extracts and plasma from unstressed (closed triangles) and stressed (open triangles) rainbow trout reaching isotopic equilibrium. Data points represent means of 2-5 fish. Sephadex bed dimensions 27.8 x 1.5 cm. Note: counts/min scale on Y-axis differs between tissues.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200
150
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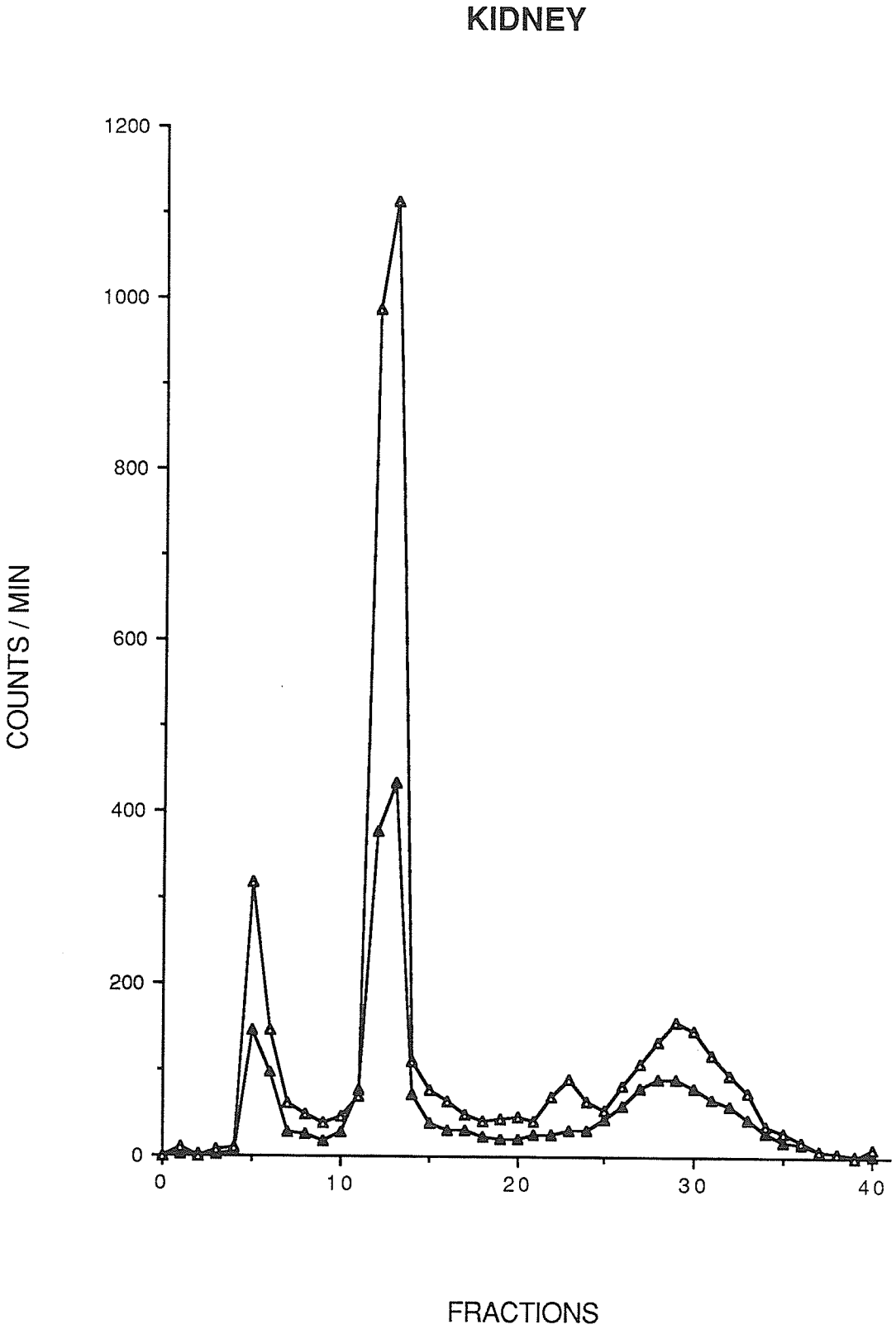
0
0
10
20
30
40
FRACTIONS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200
0
COUNTS / MIN
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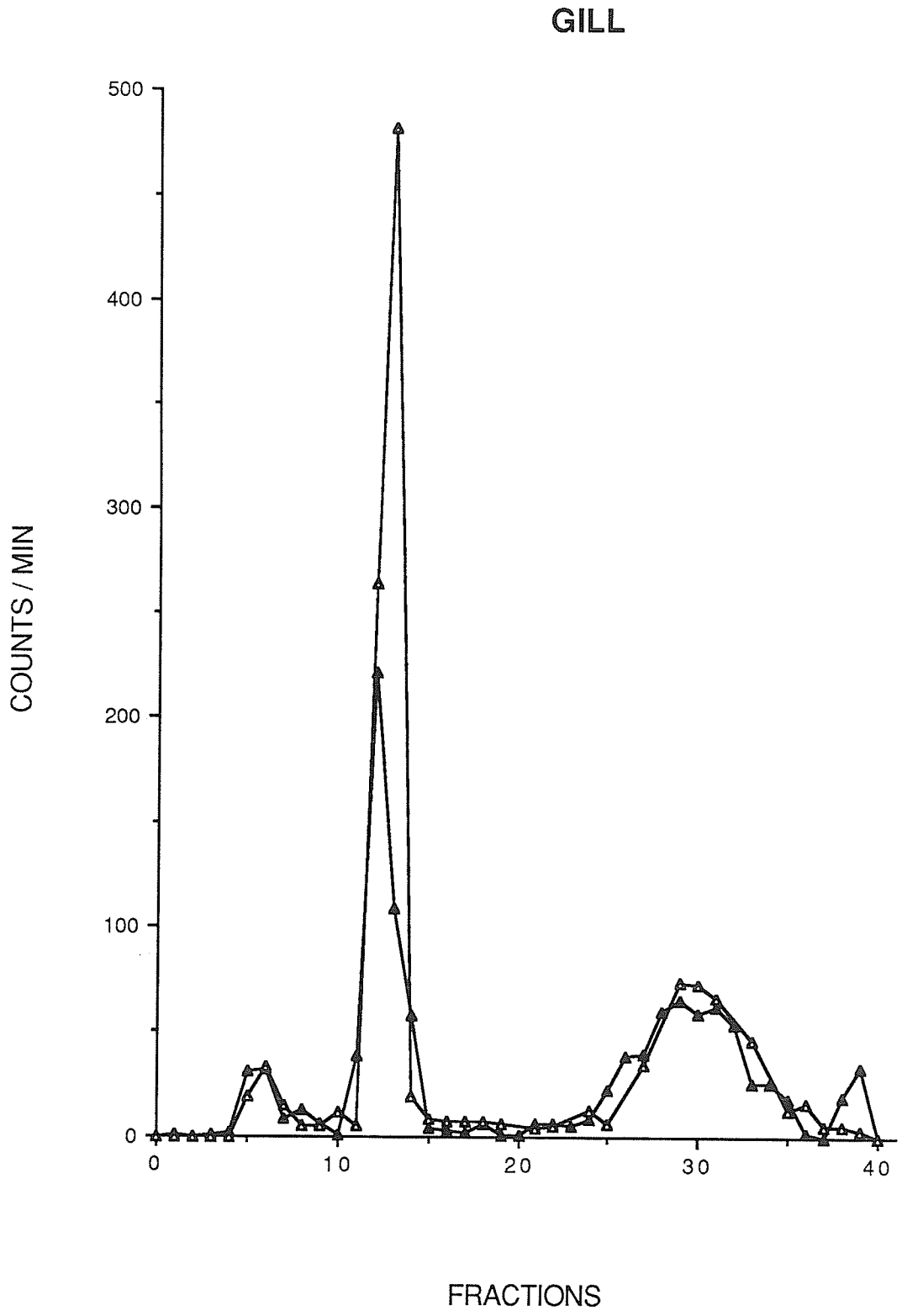
20
30
40
FRACTIONS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300
200
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0
COUNTS / MIN
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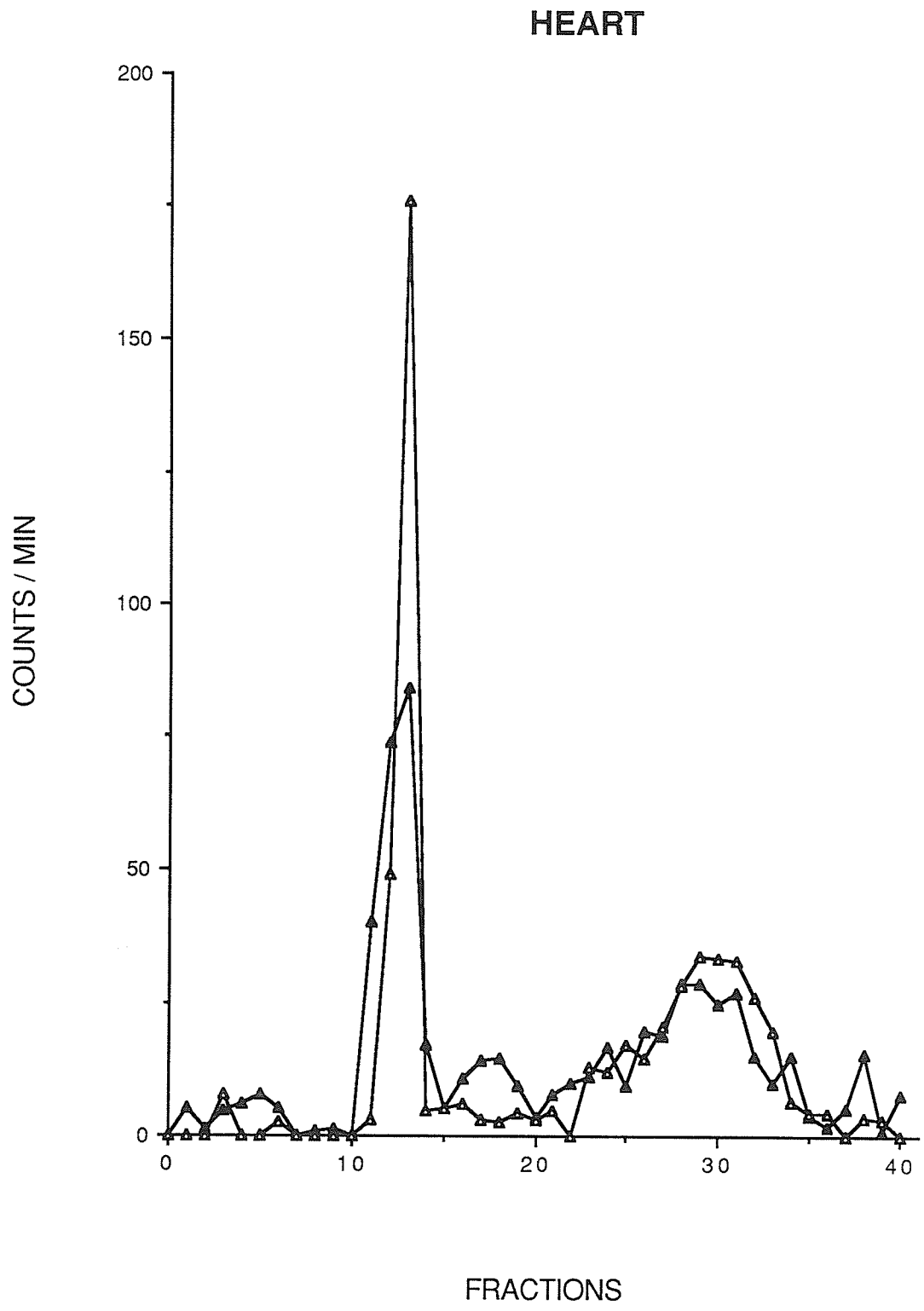
10
20
30
40
FRACTIONS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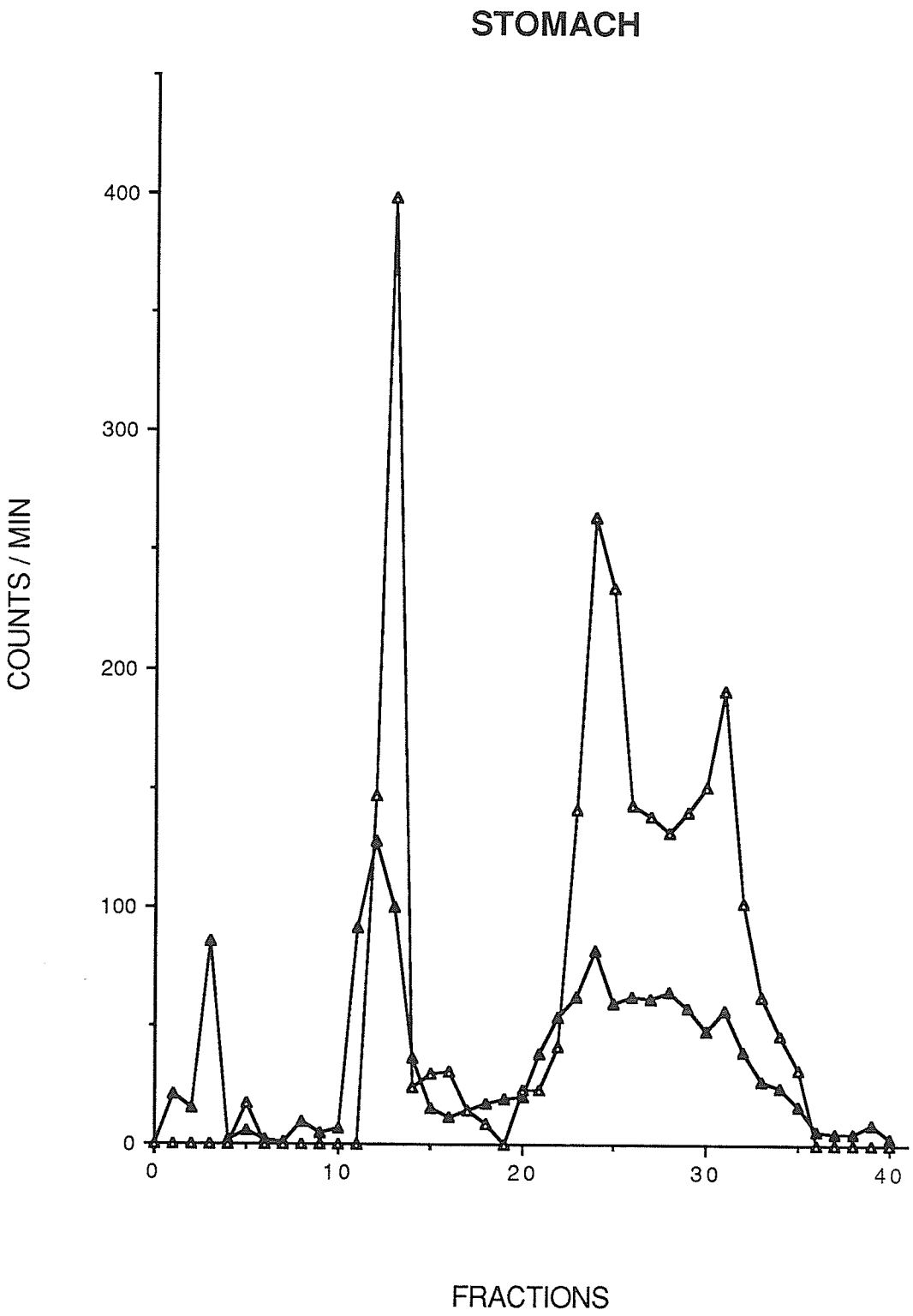
100
50
0
COUNTS / MIN
0
10
20
30
40
FRACTIONS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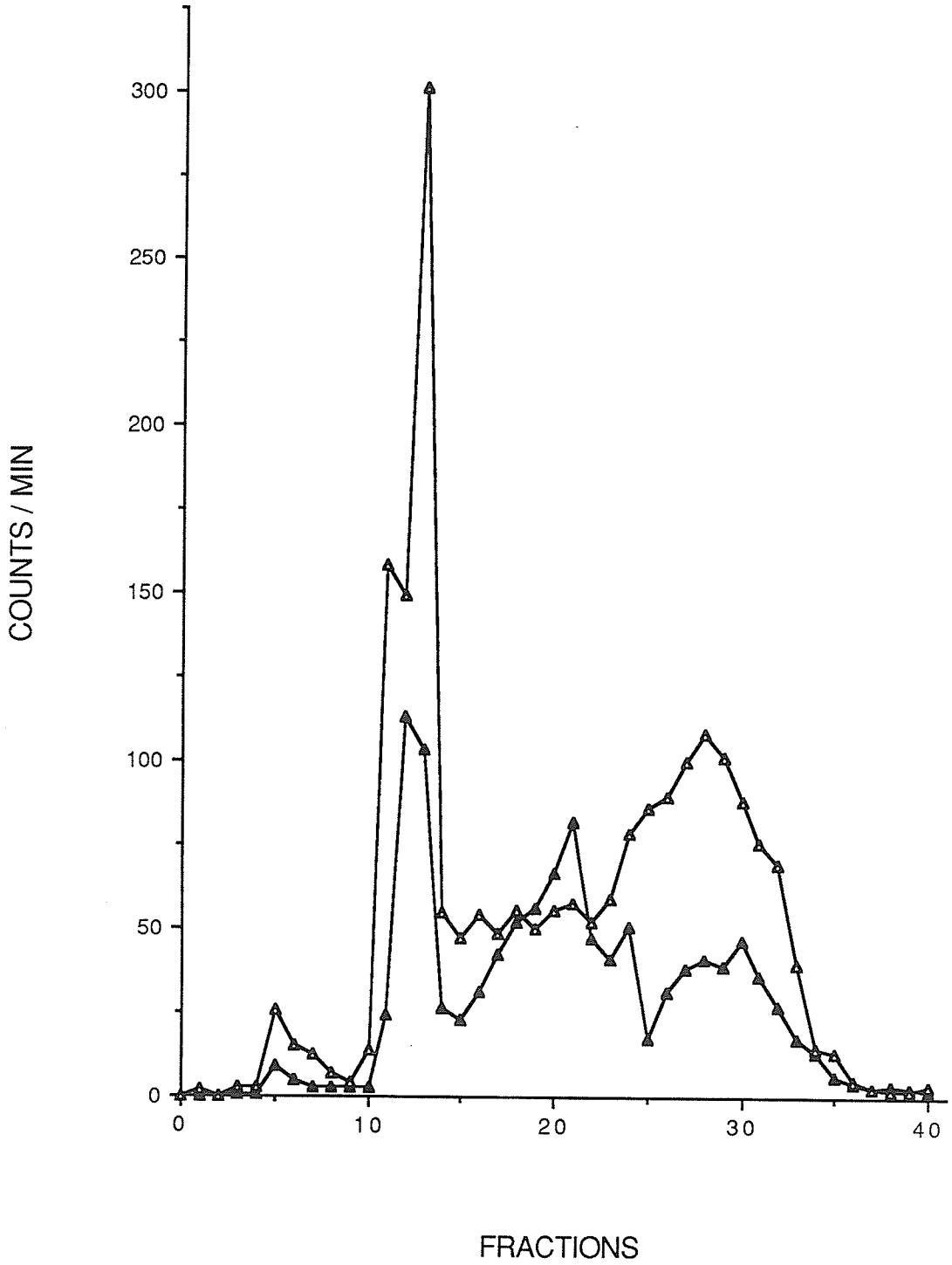
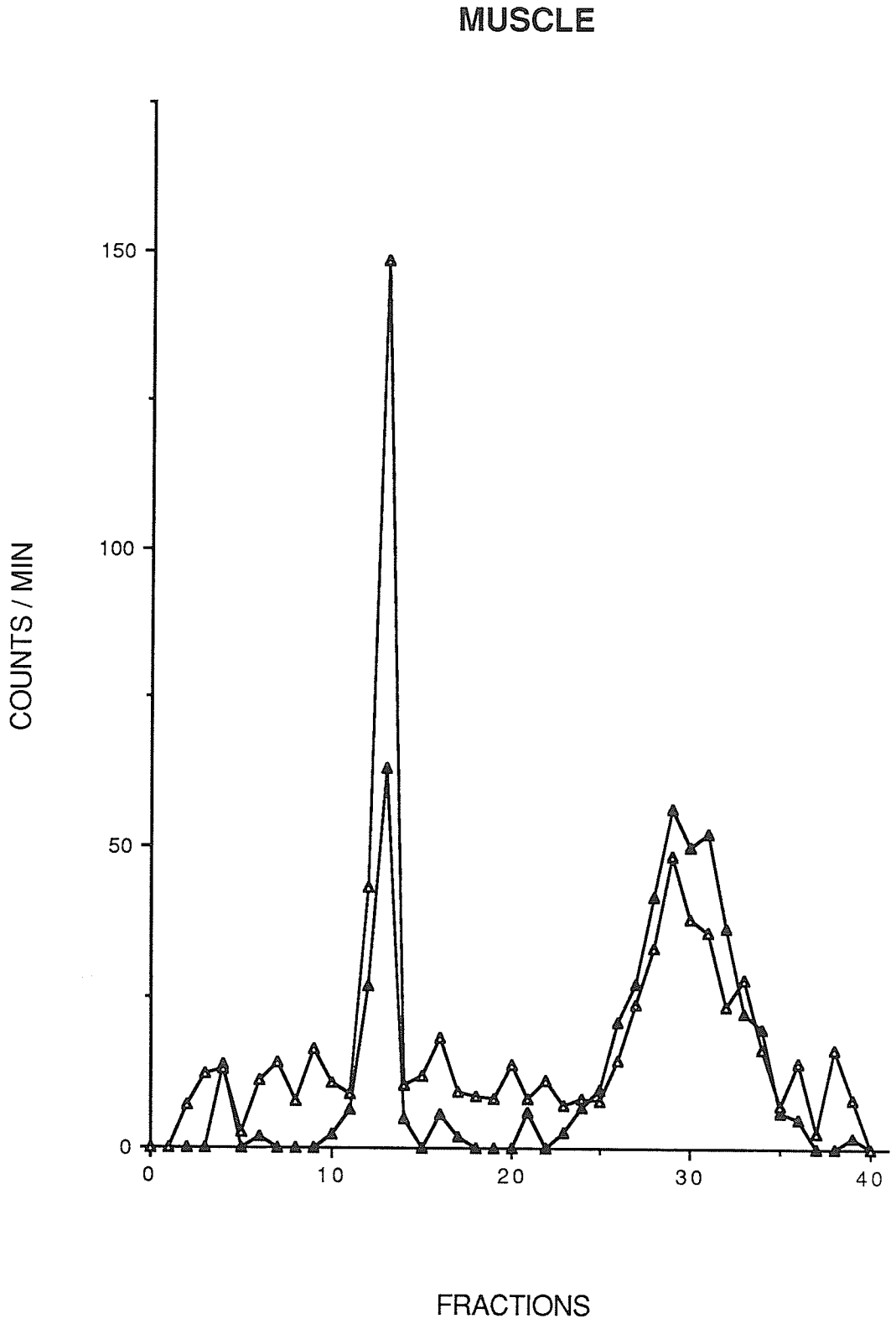
COUNTS / MIN
400
300
200
100
0
0
10
20
30
40
FRACTIONS

INTESTINE




MUSCLE
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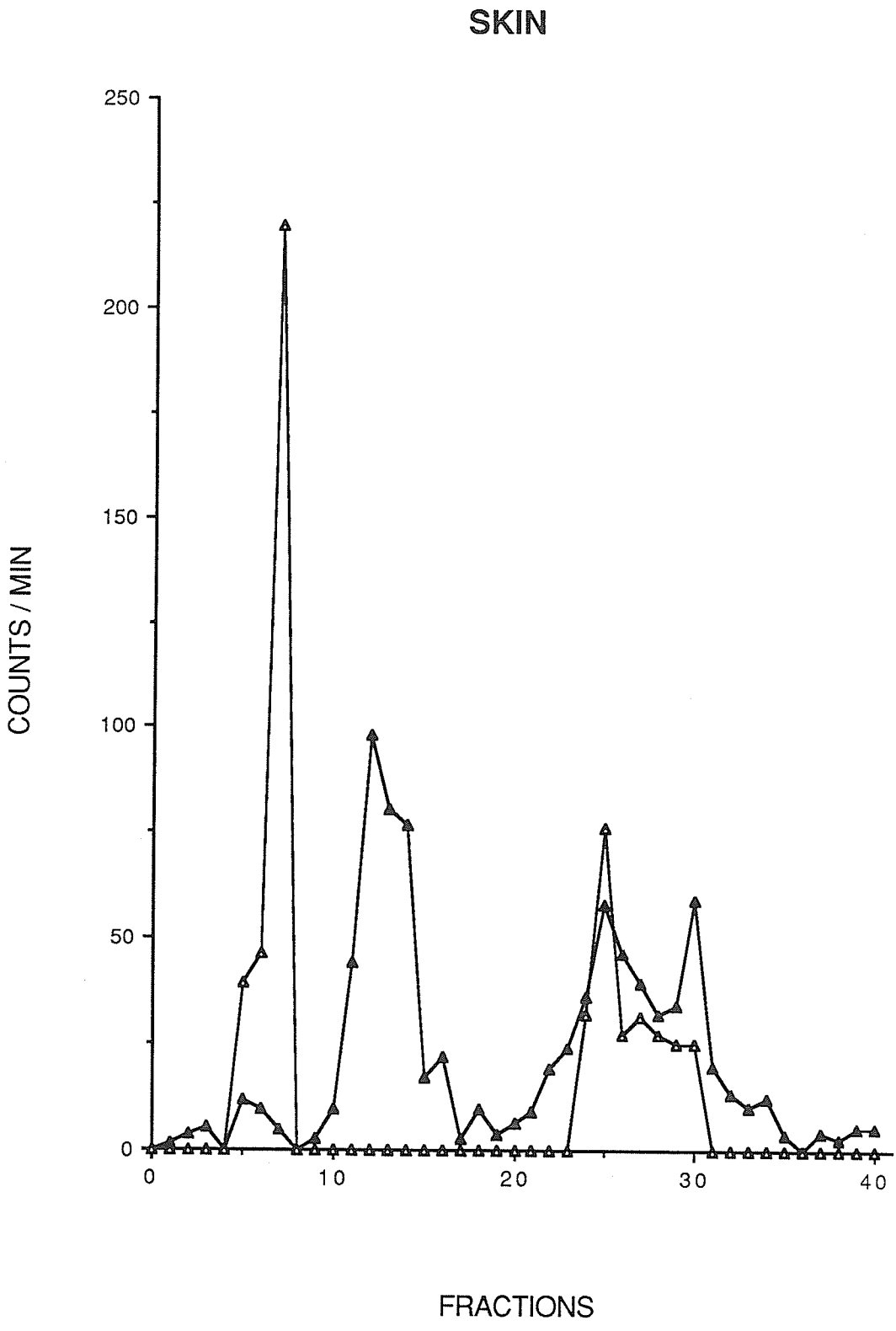
50
0
COUNTS / MIN
0
10
20
30
40
FRACTIONS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100
50
0
COUNTS / MIN
0
10
20
30
40
FRACTIONS

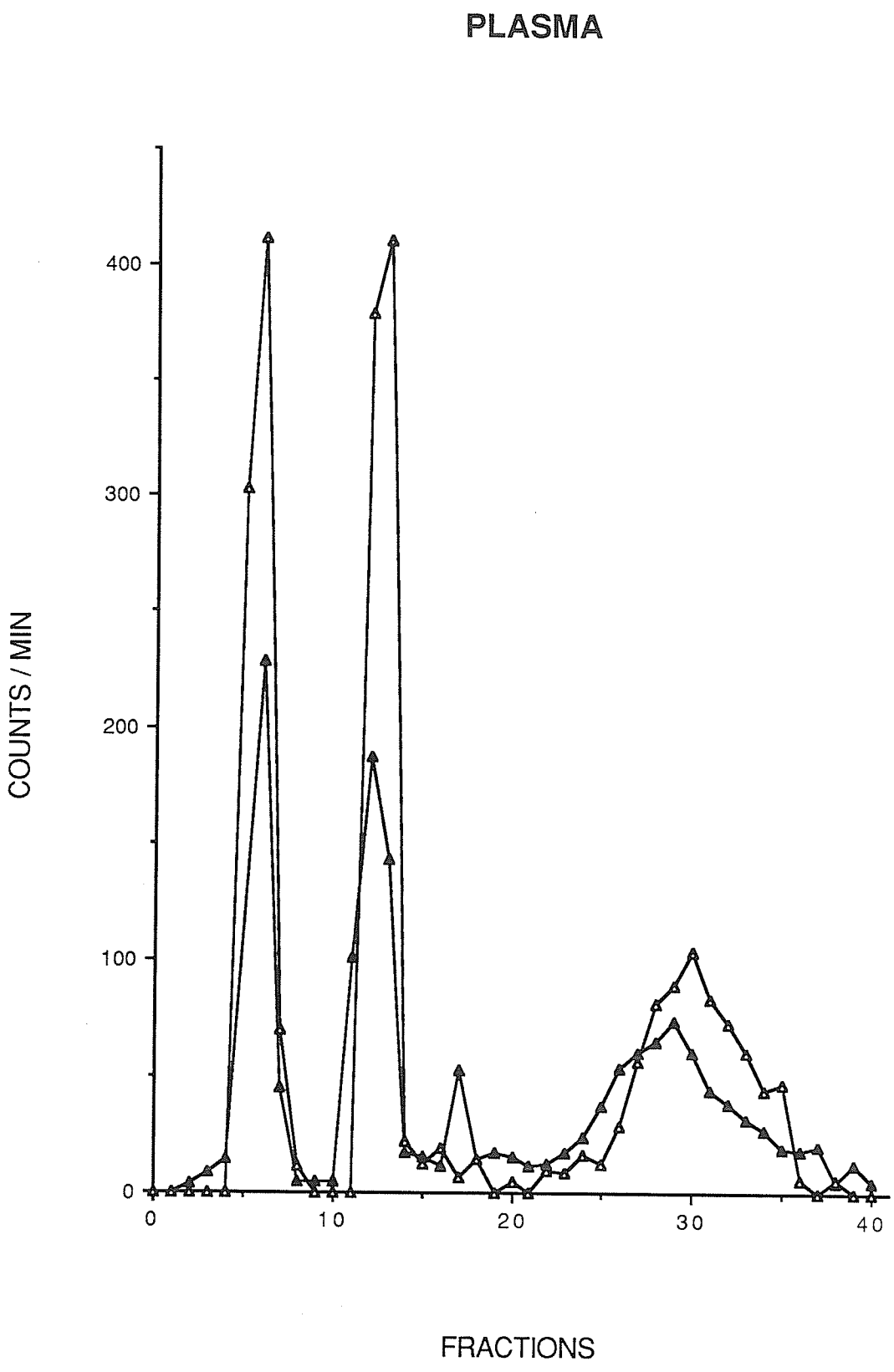

PLASMA
COUNTS / MIN
400
300
200
100
0
0
10
20
30
40
FRACTIONS

Table 5. Proportion of total radioactivity attributed to T_3 determined by chromatography, T_3 tissue concentration, and ratio of tissue to plasma (T/P) T_3 concentration for acid-stressed and unstressed trout. Values represent means (±SE) of 2-5 fish, except for skin of stressed fish, where n=1. (See App. 7-9 for individual data).

	% chromatographic radioactivity as *T_3		T_3 (ng/g tissue)		T/P	
Tissue	unstressed	stressed	unstressed	stressed	unstressed	stressed
Liver	40.7 ±7.1	36.0 ±5.9	2.46 ±0.65	0.90 ±0.26	2.31 ±0.39	1.23 ±0.25
Kidney	30.7 ±1.6	24.3 ±0.1	2.98 ±0.28	1.58 ±0.63	2.64 ±0.16	2.28 ±0.78
Gill	42.5 ±3.9	29.0 ±2.3	0.51 ±0.13	0.25 ±0.10	0.51 ±0.06	0.31 ±0.10
Heart	33.1 ±2.4	38.7 ±3.2	0.84 ±0.32	0.52 ±0.20	0.79 ±0.20	0.61 ±0.09
Stomach	36.5 ±4.2	41.2 ±3.5	1.35 ±0.65	1.25 ±0.26	1.23 ±0.45	1.19 ±0.08
Intestine	29.6 ±2.6	37.7 ±2.1	3.80 ±1.83	1.51 ±0.56	3.79 ±1.27	1.85 ±0.46
Muscle	71.2 ±2.7	37.6 ±2.6	0.61 ±0.11	0.08 ±0.02	0.54 ±0.07	0.12 ±0.02
Skin	32.8 ±1.1	44.4 -	0.27 ±0.09	0.26 -	0.28 ±0.04	0.33 -
Plasma	30.6 ±2.8	28.7 ±3.4	0.98 ±0.20	0.81 ±0.31	- -	- -

unidentified materials which would have to be identified by using elution profiles of authentic thyroid hormone analogues. For most tissues, the peaks for I^- and T_3 were separate and distinct. However, in some tissues, e.g. heart, stomach, and skin, the T_3 peak was occasionally very wide and irregular, and closely associated with an adjacent peak (Figs. 11,12,15). This was evident in the profile of skin for unstressed fish, where two sharp peaks of equal height, but not size, occurred side by side (Fig. 15). The nature of this additional peak is unknown.

For unstressed fish, differences in T_3 tissue concentrations were statistically significant between tissue types. The mean tissue T_3 concentrations were also statistically significant between unstressed and stressed fish. Intestine, kidney, liver, and stomach showed highest concentrations, whereas the amounts of T_3 in heart, muscle, gill, and skin were low. T_3 tissue concentrations of stressed fish were uniformly lower than those of unstressed fish and means did not differ significantly between tissues (Table 5). For both stressed ($p=0.01$) and unstressed ($p<0.001$) fish, T/P ratios between tissues differed significantly (Table 5). Only tissues with the highest T_3 concentrations (liver, kidney, intestine, and stomach) showed T/P ratios ≥ 1.0, indicating that in those tissues T_3 levels were higher than that found in plasma.

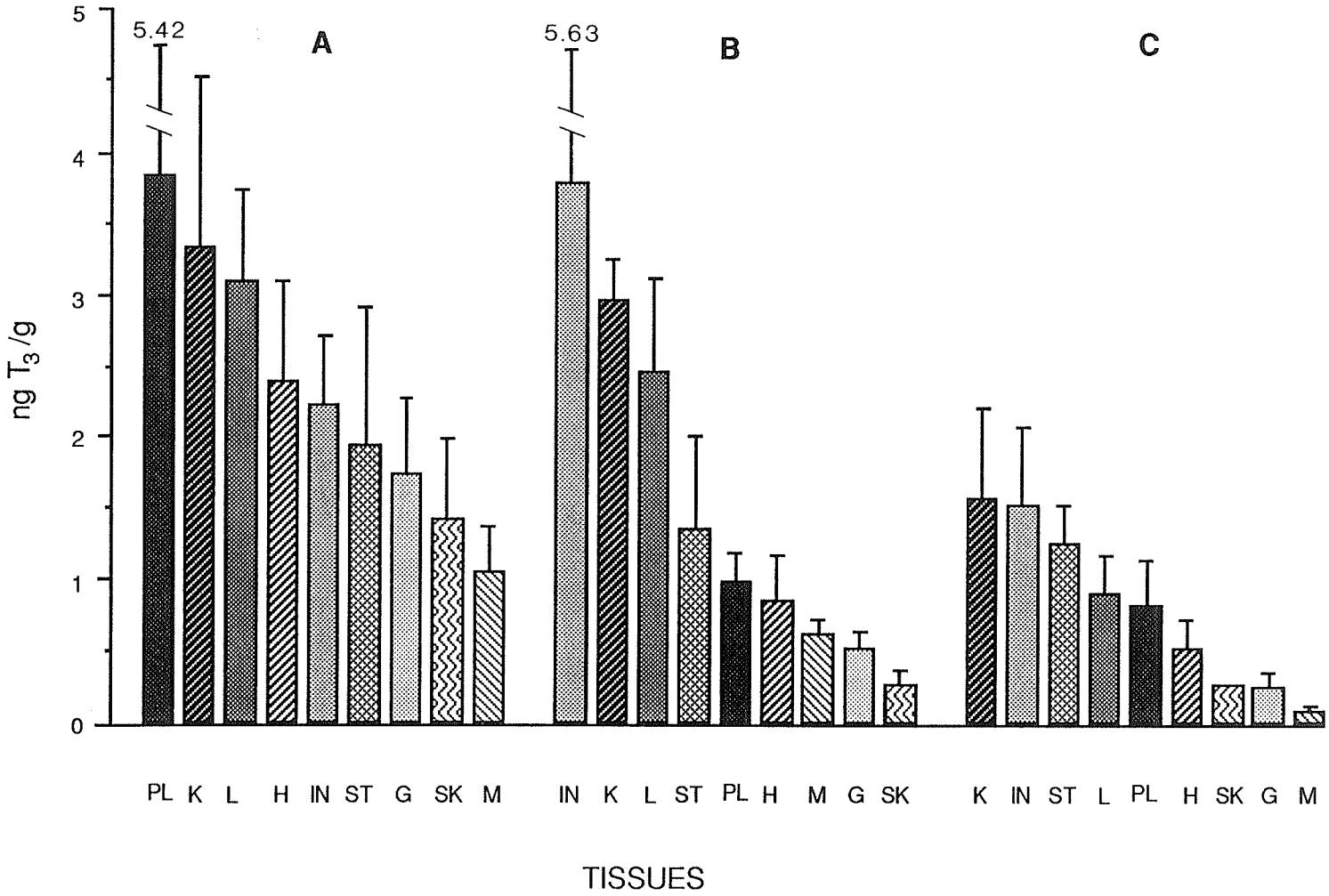
4. Ranking of Tissues by T_3 Content

When all tissues in the present study are ranked according to their T_3 concentration, tissues tend to fall into 3 groups, regardless of assay method used, or condition of the fish (i.e. unstressed or stressed), but the overall T_3 concentration was lower for the stressed fish (Fig. 17). Tissues with the highest T_3 content were intestine, kidney, and liver. Stomach and heart appeared to be intermediate, and may contain high concentrations of T_3 in individual fish. Muscle, gill, and skin tissue consistently had lower quantities of T_3. Interestingly, ranking tissue by total radioactivity/g tissue in the final 1-mL extract from fish reaching isotopic equilibrium, was very consistent in both unstressed and stressed fish; more so than ranking by actual T_3 concentrations of individuals. While the measured radioactivity likely represented substances other than T_3, it can serve an index of the relative amount of T_3 present in tissues. Plasma T_3 levels fluctuated in the ranking order, but generally had higher T_3 concentrations than those of gill, skin and muscle.

C. T_3 Content of Whole Organs

Given the concentration of T_3 per gram of tissue and the weight of the tissue, the T_3 content of tissues and organs

Figure 17. Ranking of tissue types according to T_s concentration (ng/g tissue) determined by RIA (A) and isotopic equilibrium for unstressed (B) and stressed fish (C). Bars represent means (±SE) of 3-5 fish except for skin from stressed fish, where n=1. For tissue abbreviations, see Fig.5. PL=plasma.


5
4
3
2
1
0
ng T_3 /g
5.42
5.63
A
B
C
PL K L H IN ST G SK M
IN K L ST PL H M G SK
K IN ST L PL H SK G M
TISSUES

can be determined. Furthermore, if organ growth is assumed to be allometric for a certain range in body weight, and T_3 concentration is assumed to be independent of organ weight over the same range, linear equations relating tissue/organ weights to body weight can be employed to calculate the T_3 content of organs and eventually the whole animal over the whole size range considered.

Schiffman and Fromm (1959) and Denton and Yousef (1976) regressed organ weights to body weights of hatchery-raised trout. When these regression equations were applied to the present data set, calculated organ weights either over- or underestimated actual organ weights and therefore did not appropriately describe the relationship between organ weight and body weight for the size range of fish used in this study, and also were not applicable to all organs examined. Furthermore, because of different stocks, holding conditions, and feeding regimes, size may not be a useful indicator of age, and therefore, age related differences in T_3 content cannot be accounted for.

With the exception of the intestine, the regression equations used in the present study were significant for all tissues examined (Appendix 10). For a hypothetical 300-g unstressed fish, organ T_3 content ranged from 0.5 ng for heart, to 122.6 ng for skeletal muscle. The quantity of

T_3/organ for the stressed fish is less than in the unstressed, with only slight differences between gill, heart, and stomach, but there was a difference of over 100 ng of T_3 for skeletal muscle.

DISCUSSION

A. Methodology

Key factors that were considered when devising a T_3 extraction method for this study were that the method be: (1) simple and reproducible, and (2) should work for all tissue types. These factors served as guidelines when choosing the steps of the methodology from that found in mammalian studies.

1. Enzymatic Digestion and Particle Formation

In almost all previous studies involving determination of tissue thyroid hormone concentrations, the hormones were extracted from tissue homogenates. Enzymatic digestion potentially reduces the time and effort involved in the preparation of tissues for extraction. However, it has only been applied in the extraction of thyroid hormones from homogenated thyroid glands (Rosenberg & LaRoche 1964; Inoue & Taurog 1967). Both studies report sources of error in using digestion with thyroid homogenates that affect the measurement of T_3 and T_4. The major problem was formation of particles after hydrolysis which may induce preferentially adsorb iodothyronines. Rosenberg and LaRoche (1964) found that particulate material formation depends on the duration of hydrolysis with pronase and viokase, a pancreatin enzyme.

In their studies hydrolysis with pronase for 4 h gave the maximal percentage recovery of labelled T_4, whereas 8 h of hydrolysis gave only 90-95% recovery at 4 h. For hydrolysis with viokase, 15 h seemed to be optimal. These incubation times are shorter than those used in the present method, but both the above studies used homogenized tissues to start with which may have speeded up digestion, whereas in the present study enzyme digestion was the sole method used to break down tissues thereby requiring longer incubation. Inoue & Taurog (1967) claim that under aerobic digestion, particles form after only 2 h of incubation and that shorter term incubation does not avoid the problem. They suggest that incubation under N_2 for 16 h provides better results and that under those conditions the digest contains only a small amount of particles which are dispersed by shaking to form a homogeneous suspension. Another way of reducing the amount of particulate matter in the present study, was to use tissue samples weighing 2 g or less. Flock and Bollman (1955) suggest that larger tissue samples results in a corresponding increase in the amount of interfering particulate material in the extract.

Rosenberg & LaRoche (1964) found that freezing of the hydrolysate causes aggregations of particles resulting in unequal distribution of T_4. It was first suspected that T_4 was destroyed by using prolonged hydrolysis and through

freezing and thawing samples, but the authors were able to recover the 'missing' T_4 when methanol-NH_3 extracts were made from the hydrolysates and chromatographed. T_3 losses due to freezing should not occur in the present study because the hydrolysates were refrigerated after centrifuging and never frozen. Whether the use of frozen tissue prior to hydrolysis (only fish used in constant infusion were previously frozen) affects later extraction is unknown.

Loss of radioactivity through centrifugation occurred in the present study when the initial hydrolysate was centrifuged after extraction. This left a pellet which contained 1.5-17.6% of total added radioactivity (when *T_3 was added in vitro) and 2.8-26.0% (when *T_3 was added in vivo). Comparable losses of T_4 in centrifuged compared to uncentrifuged supernatants were also reported by Rosenberg and LaRoche (1964). They concluded that the T_4 preferentially adsorbs to the hydrolysate particles and is removed from the supernatant when the particles are spun down. In the present study, the wide range in radioactivity loss after centrifugation, suggests that adsorption of radioactivity onto particles (and in turn the pellet), varies between tissues. Kidney and muscle tissue, for both in vivo and in vitro *T_3 addition, showed the highest loss. However, it was not determined if the radioactivity left in the pellet constitutes only T_3. Since centrifugation after tissue digestion

is an integral part of the extraction process, some loss of T_3 at this point is unavoidable.

The uncentrifuged hydrolysates examined by Rosenberg and LaRoche (1964) were derived from very dilute samples compared to the present digested tissues. A more appropriate comparison to their sample and results in the present study, is the effect of centrifugation on the final reconstituted extracts, which after centrifugation, showed the occurrence of preferential adsorption of hormone to particles. To avoid T_3 loss, particles in the final reconstituted extract were allowed to settle out before withdrawing samples. Aliquots contained a homogeneous amount of radioactivity compared to the proportion remaining in the test tube with the sediment. Thus, it seems likely that this sediment does not contribute to preferential adsorption of T_3.

Finally, it must also be considered that in the study by Rosenberg & La Roche (1964) only T_4 was examined. It is also unknown whether the methods and results of Rosenberg & LaRoche (1964) and Inoue & Taurog (1967) are specific for thyroid tissue or if they can be applied to other tissues.

2. Extraction Methods

In previous studies, two extraction procedures have

mainly been used, although the methods of assaying thyroid hormones varied. One of these methods is extraction by alcohol, using either butanol (Nejad et al. 1975; van Hardeveld & Kassenaar 1976), methanol (Cavalieri et al. 1984; Morreale de Escobar et al. 1985, 1986), or most frequently, ethanol (Gordon & Spira 1975; Obregon et al. 1978). Despite the variety of alcohols used, it is unknown if notable differences exist in the extraction powers of the alcohol and whether those differences depend on the type of medium from which the extraction is made (i.e. tissues or biological fluids). Method II used in the present study is a modification of Flock and Bollman's (1955) method which uses an initial alcohol extraction followed by a chloroform/ammonia wash.

3. The pH of Extractant

The quality of the extract seems to be influenced by the pH of the solution. Gross and Pitt-Rivers (1951, 1952) found that by acidifying plasma to a pH of 1-2 before extraction of iodine compounds, the quantity of thyroid hormone extracted increased, but was not qualitatively affected. This method was modified (Nejad et al. 1975; van Hardeveld & Kassenaar 1976) by addition of 2N HCl to the alcohol, rather than the plasma or tissues. The use of acidified alcohol, however, can lead to the formation of T_3 and T_4 esters in plasma and other biological fluids, which

interfere with chromatographic separation and assays for thyroid hormones (Bellabarba & Sterling 1969). Thyroid hormone esters may also form in extracts from tissues. Ester formation, however, can be reversed if extracts were made alkaline ($\geq$ pH 9.0) as soon as possible (Bellabarba & Sterling 1969).

In the present study, a further difficulty associated with the use of acidified ethanol was the formation of particles which physically interfered with the assay and which left the possibility of thyroid hormone binding to the particles making the hormone unavailable to the assay. Use of an alkaline solvent avoids the above problems and reduces possible deiodination and artifact formation in extracts run through chromatography (Boonnamsiri et al. 1979a). By using ethanolic ammonia (pH 11.4), the extract in method II was held alkaline throughout the process.

4. Compatibility of Extraction Method to RIA

Another factor to be considered in the development of an extraction method is the presence of lipid in the tissue extract. Lipids have been shown to interfere with the antigen-antibody binding in RIAs (Irvine 1974; Takaishi et al. 1978; Tagawa & Hirano 1987) as well as causing mechanical problems of clogging when used on Sephadex columns (present study). Tissue lipids will invariably appear in the

extract if only alcohol is used as an extractant. Despite the possible limitations, alcohol extraction methods have been used successfully in the determination of T_4 in eggs and larvae of coho salmon (Kobuke et al. 1987), and thyroid hormone content of various rat tissues (Gordon & Spira 1975), including skeletal muscle (van Hardeveld & Kassenaar 1978; Boonnamsiri et al. 1979a,b). In the present study, muscle was found to be one of the more fatty and problematic tissue when only ethanol extraction was used.

Interestingly, van Hardeveld & Kassenaar (1978) found that the use of ethanol alone provided better result with their particular RIA than when butanol extraction and subsequent chloroform and ammonia washes was used (van Hardeveld & Kassenaar 1976). This indicates that extraction method and RIA have to 'match' in order to obtain reliable results. Takaishi et al. (1978) compared ethanol extraction alone to butanol followed by chloroform separation based on Flock and Bollman's (1955) method for T_3 extraction from rat liver, in regards to their compatibility with various radioimmunoassays. Similar to the results in the present study, the use of ethanol alone left lipids in the extract where they interfered with the assays (Takaishi et al. 1978). Use of ethanol alone to extract T_4 from eggs of chum salmon also showed that the extracts interfered with the RIA measurement (Tagawa & Hirano 1987). These authors believed that it was

the lipoproteins and fatty acids in the yolk that caused nonspecific binding to T_4 in the RIA, as this did not occur when ethanol was used on whole fry. This problem was eliminated by using methanol and chloroform for T_4 extraction (Tagawa & Hirano 1987). Takaishi et al. (1978) concluded that a particular extraction method may not be compatible with a particular RIA, but may still be adequate for T_3 assay by a different technique.

For the trout tissues investigated and the particular RIA used, extraction with ethanol and subsequent chloroform and NH_4OH washing (method II) seemed to be most compatible. The distribution of components (water, lipids, etc.) of an ethanol extract within the chloroform and ammonia layers was investigated by Folch et al. (1957) who concluded that this distribution depended on the salt and ionic content of the tissue extract. While the second solvent extraction improves T_3 recovery, this is achieved at the cost of an additional step in the procedure and the necessity of working under the fume hood because of the potential dangers from using chloroform. Chloroform reacts vigorously with a number of common chemicals such as, fluorine, Al, Cu, N_2O_4, Na, Na/methanol, NaOH/methanol and sodium methoxide (Bretheric 1981) which must be considered in any step prior to chloroform addition.

Despite the success of using a second solvent extraction to remove lipids, subsequent validation of T_3 recovery by RIA still showed interference. To recapitulate the results, the RIA overestimated the T_3 present in a tissueless standard that had undergone extraction, but the added T_3 of an unextracted standard was accurately measured by the same RIA. These results and the inconsistent recovery of T_3 added to tissue samples suggest the presence of two antagonistic forces: (1) Both standards contained no tissues, therefore no endogenous T_3 was present to contribute to the higher than added T_3 concentration in standard 1. Possibly, a substance in the extract resembling T_3, competes with the actual T_3 for the antibody in the assay. Consequently, $*T_3$ binding to antibody is reduced, falsely indicating higher concentrations of unlabelled T_3 by the RIA. (2) RIA done on tissue samples resulted in, for the majority, a recovery of less T_3 then was added. This suggests that something in the tissues themselves causes a suppression of RIA determined T_3.

From the results of serial dilution (Fig.5), it can be shown that the substance assumed to interfere with the RIA, can also be diluted along with the actual hormones, a fact which invalidates the use of the dilution procedure as a check for the authenticity of measured T_3. This interference was not apparent in recovery of $*T_3$ by the same method

(Table 1). It was concluded that method II was not compatible with the RIA.

5. Non-extractable Compounds

When accounting for total amounts of T_3 in tissues the possible occurrence of unrecoverable or non-extractable (NE) thyroid hormones, or components of thyroid hormones (regardless of methods used), should be considered. One hundred percent recovery of T_3 may be an impossibility with tissue samples.

Surks and Oppenheimer (1970) demonstrated by injections of ^{125}I-labelled T_3 and T_4 into rats, that a fraction of the hormone radioactivity is transfered to circulating and tissue proteins. They believe that these proteins bind the residual ^{125}I that is left in tissues and plasma after alcohol extraction. Approximately 10% of the tissue organic ^{125}I was in the form of these NE ^{125}I in liver and whole body homogenates and hydrolysis of NE ^{125}I showed that it consists of ^{125}I-MIT, ^{125}I-iodothyronines, and ^{125}I, but no ^{125}I-DIT (Surks & Oppenheimer 1970). These authors assume that the hormone molecules are convalently linked to tissue structural and soluble proteins. Dratman et al. (1970) also report the presence of covalently bound ^{14}C protein fractions in tadpole tissues which are supposedly generated <u>in vivo</u> after injection of ^{14}C-T_4. Hydrolysis of the protein

fractions (similar to NE compounds found by Surks and Oppenheimer 1970) yielded ^{14}C-T_4, ^{14}C-DIT, and unidentified products believed to be formed from incomplete hydrolysis of the labelled proteins (Dratman et al. 1970).

It is interesting to note that in both of the above studies, the components of the NE fractions could only be examined after hydrolysis by pronase, the main enzyme used for tissue breakdown in the present study. Whether the use of pronase releases NE T_3 (during digestion) otherwise trapped with the other NE compounds is unknown. This suggests that the T_3 recovered by using enzyme digestion may be greater than what is recovered by the alcohol extraction process.

The IP found in chromatographs in the present study, may be a constituent of the NE fraction. Enzymatic digestion may cause a breakdown of this IP. Therefore less IP appears in tissue chromatographs than in the plasma, which did not undergo digestion and extraction. If the IP is 'non-extractable' or partially extractable, this would also explain the larger quantities of IP in plasma compared to tissues.

Surks and Oppenheimer (1970), and Dratman et al. (1970) suspected that part of NE material found in tissues may represent trapped labelled plasma proteins. This possibility

must be considered in the present study since tissues were not perfused before extraction. Present results also show that residual radioactivity, left in the pellet after centrifugation of tissue digests, and which possibly corresponds to NE material appears to be tissue-specific and reproducible. This agrees with findings by Gordon et al. (1982).

In the present study, unlabelled T_3 added *in vitro* to tissues was found to be unrecoverable when assayed by RIA. This poor recovery of unlabelled T_3, is believed not to be due to formation of NE iodothyronine compounds, but is likely a result of incompatibility of the extraction method with the RIA.

6. Determining T_3 Concentrations by $*I^-$ Infusion to IE

When continuously supplied with an external source of *I, trout did not incorporate detectable amounts of the *I into thyroid hormones. Similar results were obtained by Chan and Eales (1975) after a single injection of *I into brook trout (*Salvelinus fontinalis*). These authors speculated that this failure to detect labelled thyroid hormones was due to low plasma thyroid hormone levels and/or high plasma iodide level which caused slow radioiodide turnover. Rainbow trout also have a large plasma iodide pool with a slow turnover (Eales 1977b), but plasma iodide levels are about two-thirds

lower than for brook trout (Hunt & Eales 1979). Jacoby and Hickman (1966) successfully detected circulating iodocompounds in juvenile rainbow trout when given serial injections of Na*I until isotopic equilibrium had been reached after 56 days. This discrepency with the present study cannot be easily explained. Possibly, juvenile rainbow trout have a faster iodide turnover than adults. Furthermore, on a weight-specific basis, the total amount of *I infused in this study was, even at the highest concentration, only about 50% of the radioiodide supplied to trout in the study by Jacoby and Hickman (1966). Since most of the iodide in the plasma pool is not destined for thyroidal use (Hunt & Eales 1979), relatively small amounts of the label might not have been detected.

7. Determining T_3 Concentration by *T_3 Infusion to IE

*T_3 was directly infused into trout, and isotopic equilibrium was successfully reached. The chromatography profiles from the tissue extracts all showed distinct T_3 peaks at fractions 28-30. Another peak that occurred in nearly all tissues, was fraction 5. This pre-iodide peak of radioactivity has been observed in similar chromatographs of plasma from ^{125}I-rT_3 and ^{125}I-T_4 injected trout (Eales et al. 1983). These authors assumed that this peak was a covalently linked radioiodine in the form of iodoprotein. Its physiological significance remains obscure. Various other

peaks appeared between I^- and the T_3 peak which may represent breakdown products such as T_2 (3,3'-T_2 or 3,5-T_2), and in tissues such as liver, intestine, and kidney, conjugate products from bile and kidney, respectively, can be present.

B. Tissue Concentrations of T_3

1. Trout and Rat T_3 Concentrations Determined by RIA and IE

T_3 concentrations obtained by RIA and chromatographic analysis (IE fish), were not significantly different between the two methods (Tables 3,4, & 5). This similarity shows that although the RIA method was earlier concluded to be less accurate because of partial incompatibility with the extraction procedure, the T_3 measurements were still within the same order of magnitude. Also the relative amounts of T_3 in the respective tissues were similar.

T_3 concentrations were compared in trout and rats (literature values) for those tissues examined in both species (Table 6). T_3 concentrations in rats varied between studies, between methods (whether RIA or IE was used), and even between strains of rats (Nejad et al. 1975). Despite these differences, some generalities seem to stand out. For

both rats and rainbow trout, kidney, liver, and intestine have high T_3 concentrations, whereas muscle has the lowest values. The highest concentration of T_3 found for rat muscle (van Hardeveld & Kassenaar 1976) came from a study which only examined muscle tissue, and it is unknown whether these rats had an overall elevated T_3 level in their tissues.

In the rat, plasma T_3 concentrations are rather low, ranging from 0.16 to 0.96 ug/mL. In trout plasma, the T_3 concentrations obtained by RIA were almost an order of magnitude higher than those for rats, whereas for IE fish, T_3 concentrations were almost identical to those of rats (Table 6). (Plasma T_3 concentrations in the IE fish were also measured by RIA.) Brown and Eales (1977) determined plasma T_3 concentrations in rainbow trout of 1.3-2.0 ng/mL. An even wider range of values is not unusual as there is considerable variation in plasma T_3 in trout, depending on condition of fish or the season. It seems therefore, that levels of circulating T_3 are somewhat higher in rainbow trout than in rats, and probably mammals in general (Eales 1979).

2. Exchange of T_3 Between Tissues and Plasma

Tissue to plasma (T/P) ratios indicate the direction of gradients between the blood and the respective tissue with regards to T_3 concentration. A ratio >1.0 suggests that the

Table 6. Comparison of T_3 tissue concentrations from rat and rainbow trout determined by isotopic equilibrium (IE) or radioimmunoassay (RIA). Values represent mean T_3 concentrations expressed as ng/g tissue.

Method (Reference)	rat							trout	
	IE			RIA				IE	RIA
Tissue	(1)	(2)	(3)	(2)	(4)[a]	(4)[b]	(5)	(6)	(6)
Liver	4.50	3.92	1.90	3.85	3.05	7.69	-	2.46	3.09
Kidney	6.58	5.02	5.08	5.16	3.36	6.27	-	2.98	3.34
Heart	1.29	0.81	1.58	0.72	-	-	-	0.84	2.39
Stomach	1.16	-	-	-	-	-	-	1.35	1.94
Intestine	3.00[c]	-	3.37[d]	-	-	-	-	3.80	2.24
Muscle	0.69	0.59	0.55	0.86	-	-	1.33	0.61	1.05
Plasma	0.44	0.96	0.67	0.59	0.16	0.58	0.53	0.98	3.83

(1) van Doorn et al. 1985
(2) Obregon et al. 1978
(3) Heninger et al. 1965
(4) Nejad et al. 1975
(5) van Hardeveld & Kassenaar 1976
(6) present study

[a]Hartford rats
[b]Boston rats
[c]mean [T_3] of colon, duodenum, jejunum, and ileum
[d]small intestine only

tissue takes up T_3 from the circulation and/or forms T_3 intracellularly from T_4. Once intracellular T_3 exceeds that of the plasma, T_3 must be retained against the gradient. Conversely, a T/P ratio less than 1.0 indicates a greater concentration of T_3 in the plasma than in a tissue. In light of the uncertainties involved in the measurement of T_3 tissue concentrations by RIA, only the T/P results from isotopic equilibrium will be discussed. Mean T/P values for unstressed fish show that liver, kidney, intestine, and stomach accumulate T_3. These four tissues also consistently contained the highest concentrations of T_3. Although differences in T_3 concentrations between tissues were not statistically significant in stressed fish, differences in their T/P ratios between tissues were significant. Furthermore, the exact same tissues as in unstressed fish retained T_3 against the concentration gradient. This further supports the idea that liver, kidney, and the gastro-intestinal tract are sites of either T_4 conversion and/or uptake and storage of T_3 from the circulation.

For mammals, T/P ratios found in rats show that the concentration of T_3 in most tissues exceeds that of plasma (Obregon et al. 1978; van Doorn et al. 1985) more profoundly than in the case of trout. Similar to trout, kidney (13.94) and liver (9.35) are among the tissues with the highest T/P ratios (van Doorn et al. 1985), suggesting that these two

organs assume special importance for peripheral TH metabolism in all vertebrates.

T_3 accumulation may be accomplished by various T_3 translocation mechanisms at the cellular level. Evidence from in vitro studies suggest that iodothyronine transport is assisted by plasma-membrane proteins (Oppenheimer & Surks 1974; Rao et al. 1976; Eckel et al. 1979; Pardridge 1984).

The accessibility T_3 has into a tissue may also be reflected in the amount of T_3 found in a particular tissue. A factor that determines the exchange of T_3 between plasma and tissue is the permeability of the endothelium of the vessels bathing the tissue. The morphology of capillaries differs in endothelium structure and cell to cell junctions, and there is evidence for regional differences in permeability among capillaries of the same morphological type (Bloom & Fawcett 1975). The endothelium lining inside the liver has relatively large gaps which allow close contact between plasma proteins and the cell membranes of the hepatocytes (Pittman 1979). In muscle the less protein-permeable vascular endothelium may account for the slow plasma-tissue T_3 exchange rate (Oppenheimer 1983), therefore low quantities of T_3 entry and low T_3 concentrations.

Transport of T_3 may not only be tissue-specific, but is

influenced by whether the tissue is studied *in vitro* and *in vivo*. This may explain why in the present study, recovery of a *T_3 source added *in vitro*, differed from that of *T_3 incorporated by the fish *in vivo* (Table 1). Differences in *T_3 recovery between tissues *in vivo*, indicate that T_3 distribution and incorporation may differ between tissue types. Extraction variability possibly depends not only on T_3 entrance into tissues, but also on the intracellular T_3 binding ability of a tissue. Once T_3 enters into the cell most of the hormone becomes associated with cellular components such as organelles, membranes and soluble proteins, and only a small portion of intracellular T_3 exists in free form (Cavalieri & Pitt-Rivers 1981). Another way for tissues to establish and to retain a T_3 concentration gradient against the plasma, is to bind available T_3 to these intracellular binding sites, thus 'trapping' any free or incoming T_3. It is unknown if the number or types of binding sites is specific for a tissue type, but if T/P ratios of T_3 concentrations represent a useful indicator, at least some cell types of kidney, liver, and intestine may have larger numbers of such sites.

3. Fast and Slow Equilibration Tissue Pools

T/P ratios and T_3 concentrations represent a static picture of the relationship between tissue and plasma concentrations of thyroid hormones. The T_3 content of whole

organs and tissues can be compared with estimates of T_3 distribution found by kinetic studies which examine the TH exchange rate between plasma and cellular pools.

From studies with rats, DiStefano et al. (1982a) developed a kinetic model based only on plasma measurements, which breaks down the distribution of T_3 into three pools. Of the total body T_3 pool, 3-4% is found in the plasma, 76% is contained in what is termed slowly equilibriating (slow) tissue pools, characterized by tissues such as muscle, skin, and brain. The remaining 19-21% is contained in rapidly equilibrating (fast) tissue pools, typified by liver and kidney (DiStefano 1982, DiStefano et al. 1982a). Tissues such as heart and pituitary are considered intermediate in their rate of equilibration (Oppenheimer 1983). This relative distribution of T_3 in rat tissue is nearly identical to that in man, when the data are normalized for body weight or when calculated as a percentage of total quantity (DiStefano 1982).

In rainbow trout, the categorization of fast and slow equilibrating tissues has also been attempted following cardiac injection of $*T_3$ (Van Der Kraak 1979). Tissues that followed the distribution of $*T_3$ in plasma were liver, kidney, and gill and were considered fast equilibrating tissues. Slow equilibrating tissues such as intestine,

spleen, stomach, skin, muscle, and gall bladder, were characterized by a period of increased uptake despite decreasing plasma *T_3 (Van Der Kraak 1979).

In order to compare the relative distribution of fast and slow tissue pools and to compare the total body T_3 pool in trout, to those found in rats, calculations were based on organ T_3 content for a hypothetical 300 g unstressed fish. The combined amount of T_3 from liver, and kidney (i.e. fast pool for rats) equals 15.4 ng (Table 7). Assuming that the fast pool in trout makes up 20% of the total T_3 body pool, as has been found in rats (DiStefano et al. 1982a), the body T_3 pool would contain 77 ng. Muscle tissue, a component of the slow tissue pool, accounts for 67% of body weight in adult rainbow trout (Stevens 1968), and muscle T_3 alone contains 123 ng in a 300 g fish. Obviously the T_3 body pool must be substantially bigger than the above 77 ng estimate. From these theoretical considerations, it can be concluded that if fast and slow pools comprise similar tissues in rainbow trout and rat, the percentage contribution of the two pools to the total body pool of T_3 must differ between these animals. This can be at least partially explained by the somewhat lower concentration of T_3 in both liver and kidney of rainbow trout than that found for rats and the larger proportion of muscle tissue in salmonids.

Table 7. Amount of T_3 (ng)/organ of a hypothetical 300g trout. Organ weights are calculated from organ to body weight regressions (see App.10). T_3 concentrations were taken from fish reaching isotopic equilibrium. Muscle weight was taken as 67% of body weight.

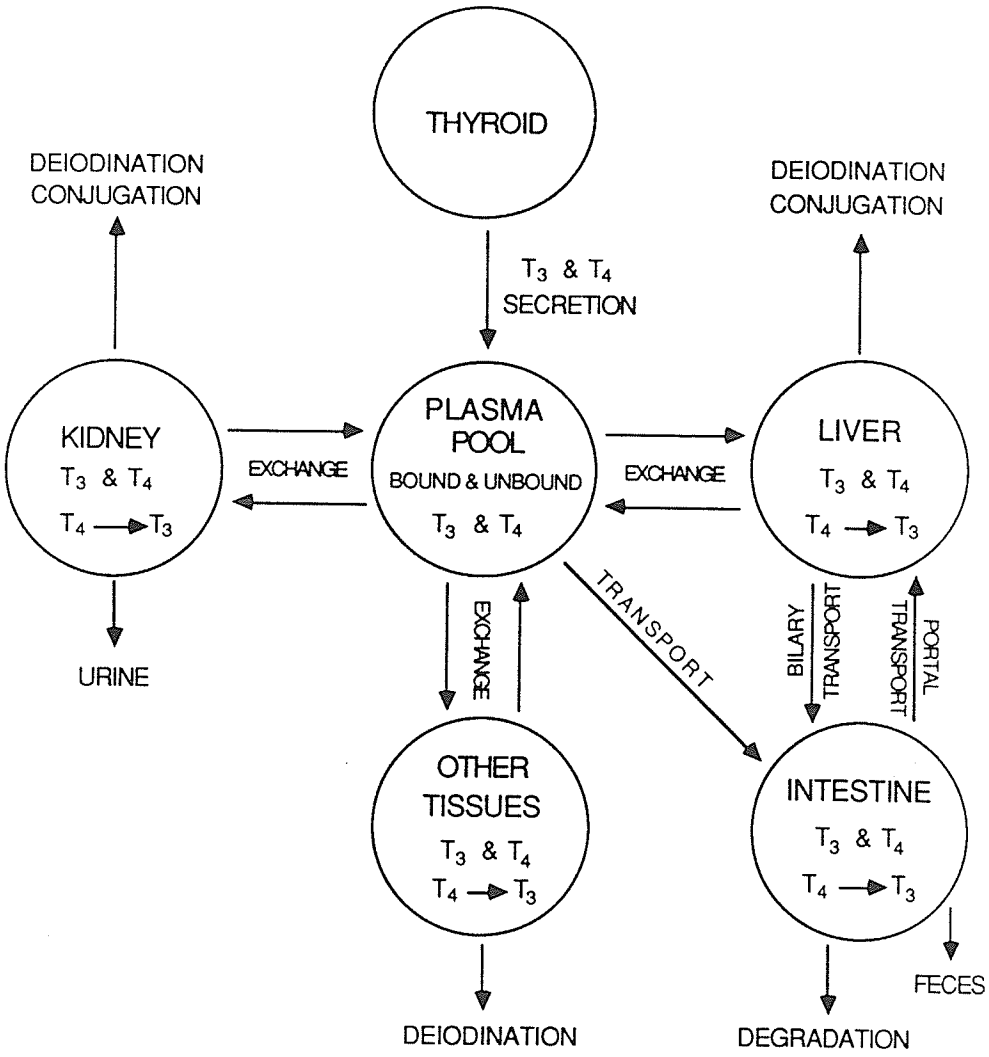
Tissue	Organ weight (g)	T_3 (ng/g tissue)		T_3 (ng/organ)	
		unstressed	stressed	unstressed	stressed
Liver	3.58	2.46	0.90	8.81	3.22
Kidney	2.21	2.98	1.58	6.59	3.49
Gill	3.70	0.51	0.25	1.89	0.93
Heart	0.60	0.84	0.52	0.50	0.31
Stomach	2.69	1.35	1.25	3.63	3.36
Intestine	1.52	3.80	1.51	5.78	2.30
Muscle	201.00	0.61	0.08	122.60	16.08

4. Sources of T_3

The sources of T_3 available to a tissue must be considered, as they may ultimately determine the T_3 concentration of that tissue. Figure 18 summarizes the major sites and pathway of TH metabolism in mammals. It shows that apart from receiving T_3 directly from the thyroid (still questionable for fish), most mammalian tissues are capable of deiodinating T_4 to T_3 (Chopra 1977). This T_3 is either released into circulation where it enters the plasma pool or remains within the tissue, forming a further source of T_3 at the cellular level. In mammals, between 20% (Schwartz et al. 1971) and 66% (Silva et al. 1982) of total T_3 are obtained through deiodination. Studies with rats have shown that liver and kidney produced the bulk of circulating T_3 (Chopra 1977; Kaplan 1983; van Doorn et al. 1983). In rainbow trout, as much as 70% of injected $*T_4$ undergoes deiodination to T_3 (Eales 1977b), indicating that the production of extrathyroidal T_3 is at least of similar magnitude to that in mammals.

Although deiodination of T_4 has been established in teleosts, not much is known about the contribution of specific tissues to the T_3 pool. Law and Eales (1973) documented $*T_4$ deiodination to *I (but not $*T_3$) in homogenates of brook trout gill, intestine, heart, and brain, but not in liver. In rainbow trout, however, the deiodination and

Figure 18. Physio-anatomical model of T_3 sources, distribution, and excretion in mammals. (Modified after DiStefano 1984).


THYROID
DEIODINATION
CONJUGATION
T_3 & T_4
SECRETION
DEIODINATION
CONJUGATION
KIDNEY
T_3 & T_4
$T_4 \rightarrow T_3$
EXCHANGE
PLASMA
POOL
BOUND & UNBOUND
T_3 & T_4
EXCHANGE
LIVER
T_3 & T_4
$T_4 \rightarrow T_3$
URINE
EXCHANGE
TRANSPORT
BILARY
TRANSPORT
PORTAL
TRANSPORT
OTHER
TISSUES
T_3 & T_4
$T_4 \rightarrow T_3$
INTESTINE
T_3 & T_4
$T_4 \rightarrow T_3$
FECES
DEIODINATION
DEGRADATION

the formation of T_3 has been shown to occur in liver (Pimlott & Eales 1983; Shields & Eales 1986), and kidney homogenates, but not in spleen, skeletal muscle or gill (MacLatchy, unpublished data).

Imai et al. (1981) has shown in rats, that there is a positive correlation between the deiodinating activity of a tissue and the T_3 concentration. In the present study, the same correlation may exist as liver and kidney have a relatively high T_3 concentration, whereas muscle and gill concentrations fall in the lower range of all examined tissues. This is further supported by the fact that in the livers of acid (pH 4.7) and aluminum (20 µM) exposed rainbow trout, deiodinase activity is greatly reduced (MacLatchy, unpublished data). The reduction in T_4 deiodination may be reflected in the tissue T_3 concentration, which in stressed fish were lower than unstressed fish (present study).

The ability of certain tissues to regulate local T_3 levels independent of plasma T_3 is also indirectly supported by the present result of tissue specific T_3 concentrations and may represent an adaptive mechanism to regulate the quality and quantity of TH action.

With regard to the fate of locally generated T_3 and its contribution to intracellular T_3, considerable differences

seem to exist between tissues. Most of the T_3 produced from conversion of T_4 in rat liver and kidney reenters the circulation and accounts for approximately two-thirds of plasma T_3 (van Doorn et al. 1983). However, whereas only a minor portion of T_3 within kidney cells stems from local conversion of T_4 to T_3 directly, in the liver this source still amounts to 40% of total tissue T_3 (van Doorn et al. 1983, 1985). In muscle, this portion of total T_3 was only minor since T_3 was found to be derived solely from plasma (van Doorn et al. 1982). It is unknown if the same partitioning of locally generated T_3 also occurs in fish tissues. This may explain high T_3 concentrations in the liver of trout, but in kidney, stomach and intestine, the T_3 may also be derived from plasma alone. It is possible, therefore, that liver serves as a supplier of T_3 for the rest of the tissues by its deiodination activity.

5. Physiological Significance of TH and Tissues

A general overview is given of tissue types examined in the present study and their role in physiological interactions with thyroid hormones in both teleosts and mammals. The majority of circulating hormones enters tissues which serve in excretion, degradation or metabolism of the hormone, but may not necessarily be a target site.

Skeletal muscle represents the largest thyroid-hormone-

sensitive tissue in mammals, and therefore may assume an important role in overall thyroid hormone metabolism. It is unknown if skeletal muscle is TH-sensitive in teleosts. The effect of thyroid hormones on skeletal muscle in mammals is linked with regulating nonshivering thermogenesis. A comparable effect in poikilotherms has not yet been described and probably does not exist. T_3 formed locally from T_4 has been demonstrated in rat skeletal muscle (van Hardeveld & Kassenar 1978), but the rate of T_4 conversion to T_3 did not correspond to the higher rate of T_3 muscle uptake. Variations in uptake rate were further complicated by whether the muscle was of the red or white type (van Hardeveld & Kassenar 1978). Although muscle tissue has low T_3 concentration, when compared to the other tissues (Table 5), its dominating contribution to total body mass makes it the single largest source of T_3, possibly acting as a storage site. Rat muscle tissue comprises approximately 50% of total body weight (DiStefano et al. 1982b); in rainbow trout, this percentage is even higher, at approximately 67% of total body weight (Stevens 1968).

Skin in teleosts has been reported to show a wide range of responses to THs. For example, color changes can be induced by increasing deposition of guanine crystals which increases silvering (Matty 1985), or by altering melanophore densities (Eales 1979). Skin and gill are possible sites of

T_3 accumulation because both tissues may act as excretion and/or absorption pathways. Such transport of T_3 is plausible because of the large surface area of skin and gills and their proximity to the environment. The existence of the above pathways has been indirectly supported by observations that T_3 can be introduced into rainbow trout via immersion into water containing T_3 (Eales & Collicutt 1974; Omeljaniuk & Eales 1985). Under those conditions, absorption of T_3 may have occurred via the GI tract, but this route has been considered negligible for freshwater fish (Eales & Collicutt 1974). Present T_3 concentrations in skin and gills were among the lowest for any tissue measured. While these results indicate that neither gill or skin act as storage sites for T_3, they do not exclude the possibility that these two tissues act as exit or entrance way for T_3 without any accumulation at the site. It remains unknown whether the teleost skin acts as a site for deiodination of T_4 to T_3 as has been suggested for humans (Hays & McGuire 1980).

In the mammalian heart, a relationship exists between catecholamines to cardiovascular changes (e.g. increased cardiac output, accelerated heartbeat) in altered thyroid states, although it is not completely understood at what level the interaction between TH and catecholamines take place (Freedberg & Hamolsky 1974). TH has been found to increase the number of β-andrenergic receptors in rat heart,

but this increase in receptors appears to be species specific (Morkin 1984). No parellel action has been investigated in teleosts.

Not much is known about direct TH interactions with the stomach in either mammals or teleosts. It is unknown if T_3 is deiodinated in the stomach along with ingested food or if it is absorbed before being passed into the intestine where absorption of T_3 can occur (van Middlesworth 1974). The relatively high T_3 concentrations of stomach tissue found in the present study tentatively suggest that some storage of T_3 takes place, which may be peripherally generated or absorbed from the food.

Liver and kidney are generally considered responsive to TH and are the tissues most often studied due to their large size, and high T_3 concentration (Table 6). Both are also sites of active deiodination of T_4 and are considered to be a major source of T_3 in mammals (Chopra 1977; van Doorn et al. 1982). These organs represent routes through which THs are broken down, conjugated to glucuronides and sulphates, and excreted as bile or reabsorbed (Galton & Pitt-Rivers 1959; van Middlesworth 1974; Pittman 1979; Fig. 17). Although T_3 in bile was not examined in the present study, bile was considered to be a contaminant in liver and intestinal tissues.

A bilary excretion route for T_4 and T_3 has also been demonstrated for brook trout (Eales et al. 1971). In the present study, fish were starved to reduce entry of bile into the intestine. Rainbow trout that received an ambient source of $*T_3$, showed that discharge of radioactive bile into the intestine after two days of starvation contributes only negligiblly to intestinal radioactivity (Eales & Collicut 1974). Nevertheless, the present study still found bile present in the saline solution after rinsing, indicating that bile may still be released and retained in the intestinal lining after starvation. Therefore, bile may still be carried through the extraction procedure along with the intestine extract.

The presence of bile containing an assortment of hormones and conjugates, as well as the possibility of TH analogues formed within the intestine, may explain the presence of the very large peak in the corresponding chromatograph for unstressed fish (Fig. 13), possibly indicating the presence of T_1 or T_2. The high concentration of T_3 in the intestine may also reflect the possibility of T_3 formation and reabsorption.

Although enterohepatic cycling of T_3, and T_3 absorption from the intestine has not yet been demonstrated in fish, studies on rats have shown that when $*T_3$ is introduced by

cannulation, 43% of the *T_3 entering the gut with the bile was excreted as *T_3 in the feces. The remaining 57% were either reabsorbed and transported to the liver via the enterohepatic circulation or degraded in the gut (Sternlicht et al. 1984). Similarly, hydrolysis of iodothyronine conjugates into T_1, T_2, T_3, rT_3, and T_4 in the intestine has so far only been documented for mammals, where the reaction seems to depend on the presence of bacteria and is possibly not enzymatic in nature (de Herder et al. 1974).

SUMMARY

1. Use of alcohol alone (method I) or acidified alcohol as an extractant, was not useful for fatty tissues, as lipids were extracted along with T_3.
2. Use of a chloroform/ammonia wash after alcohol extraction (method II) removed lipids and gave a recovery of added *T_3 ranging from 55.8% (kidney) to 83.8% (heart).
3. Recovery of unlabelled T_3 (measured by RIA) was inconsistent and it was concluded that the extraction method is incompatible with the RIA method.
4. T_3 concentrations obtained by an alternate procedure involving chromatography of tissue extracts from fish receiving constant *T_3 infusion until isotopic equilibrium was reached generally fell into 3 groups: highest T_3 in intestine, kidney and liver; intermediate T_3 in heart and stomach; lowest T_3 in gill, muscle and skin. The relatively high T_3 concentration in kidney, liver and intestine, and low muscle T_3 concentration are similar to literature values for rats.
5. Tissue-plasma T_3 concentration ratios were significantly different between tissue types, indicating differences in T_3 metabolism between tissues.
6. T_3 concentrations of tissues from acid or acid and aluminum stressed trout had the same relative distribution

as did tissues of unstressed fish, but absolute T_3 concentrations were significantly lower.

7. T_3 contents of organs of a hypothetical 300 g stressed trout were lower than those for an unstressed fish, the quantity of T_3 of skeletal muscle being 7 times lower in stressed fish.
8. In conclusion, it was possible to (a) measure the T_3 concentration in various tissues of the trout, (b) show that differences do exist between tissue types and (c) show that the T_3 concentration can change due to the physiological state of the fish.

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APPENDICES

Appendix 1. Specific activity calculated from plasma values at time of death and used for determining T_3 concentration in tissue extracts. Fish 1-5 were unstressed, fish 6 was acid stressed (pH 4.8), and fish 7-8 were acid and aluminum stressed.

Fish	Plasma T_3 (cpm/ml)	Plasma T_3 (ng/ml)	Specific Activity (cpm/ng)
1	2052.09	1.35	1398.46
2	2492.90	1.10	2084.97
3	6077.45	0.78	7261.37
4	3535.67	0.30	1730.23
5	3400.42	1.35	1496.84
6	4117.34	0.77	4856.35
7	2867.56	1.88	8793.85
8	3354.38	2.09	2285.95

Appendix 2. Recovery of T_3 from homogenized and enzymatically digested liver and kidney. T_3 cpm determined by short column separation. Values represent means (±SE) of 3 fish.

	homogenized		digested	
Supernatant	T_3 cpm	% of total	T_3 cpm	% of total
		a) Liver		
1	130.1 ±11.1	35.8 ±2.5	153.6 ±28.6	43.8 ±6.4
2	107.4 ±12.2	29.3 ±1.7	128.4 ±29.7	36.4 ±6.9
3	53.5 ± 3.2	14.8 ±1.4	34.8 ± 8.7	9.6 ±1.7
4	29.8 ± 5.1	8.2 ±1.4	15.9 ± 3.2	4.7 ±1.3
5	22.6 ± 3.0	6.2 ±0.9	10.5 ± 1.5	3.1 ±0.7
6	20.6 ± 0.5	5.7 ±0.4	8.2 ± 1.4	2.5 ±0.7
		b) Kidney		
1	46.9 ±13.7	42.6 ±4.8	41.1 ±11.8	36.6 ±5.6
2	15.7 ± 4.3	14.7 ±3.4	21.2 ± 6.2	19.0 ±3.2
3	12.8 ± 3.4	11.6 ±0.9	16.5 ± 5.0	15.1 ±3.7
4	11.3 ± 1.6	10.8 ±0.8	12.0 ± 1.4	12.0 ±3.1
5	10.4 ± 0.5	10.3 ±1.7	8.2 ± 2.2	7.7 ±1.7
6	10.1 ± 0.9	9.9 ±1.3	9.4 ± 1.5	9.7 ±3.2

Appendix 3. Percent radioactivity recovered after extraction from rainbow trout tissues after *in vitro* and *in vivo* addition of $*T_3$.

	in vitro			*in vivo*		
Tissue	Fish 1	Fish 2	Fish 3	Fish 1	Fish 2	Fish 3
Liver	-	66.8	67.6	73.6	76.5	75.6
Kidney	-	72.3	62.4	59.1	53.7	54.5
Gill	-	63.2	75.8	87.5	77.9	83.0
Heart	-	66.0	79.4	82.6	94.8	74.0
Stomach	79.7	63.0	74.2	93.3	77.2	72.0
Intestine	72.2	63.9	85.8	88.2	88.5	69.9
Muscle	65.7	63.0	61.9	71.6	69.8	63.6
Skin	74.1	67.9	76.9	79.3	72.5	66.0

Appendix 4. T_3 tissue concentrations (determined by RIA) after 3 serial dilutions of the extract. Values represent mean (+SE) percentages. 100% corresponds to the undiluted sample.

Tissue	n	Dilution		
		50%	25%	12.5%
Liver	8	53.0 ±1.7	27.9 ±1.6	13.9 ±0.9
Kidney	8	58.1 ±2.5	30.9 ±2.0	15.3 ±0.4
Gill	8	55.5 ±2.0	28.0 ±4.1	16.5 ±4.5
Heart	7	53.1 ±3.4	30.2 ±5.5	22.7 ±9.0
Stomach	9	48.3 ±3.3	28.1 ±3.0	16.5 ±2.8
Intestine	9	54.6 ±3.5	28.9 ±1.7	14.5 ±1.3
Muscle	9	43.8 ±1.6	29.7 ±4.5	10.9 ±2.2
Skin	9	56.4 ±3.0	26.1 ±3.3	19.8 ±4.4

Appendix 5. T_3 tissue concentrations (determined by RIA) after 3 serial dilutions of extracts from tissues with and without an added T_3 dose. Values represent mean (+SE) percentages. 100% corresponds to undiluted samples.

	without T_3			with T_3		
Tissue	50%	25%	12.5%	50%	25%	12.5%
Liver	56.1	28.3	13.9	47.9	25.7	12.7
Kidney	58.1	27.4	12.6	55.0	26.4	18.0
Gill	64.3	28.8	16.8	52.9	29.5	15.3
Heart	29.8	16.0	5.8	54.8	30.0	15.5
Stomach	53.4	23.9	10.7	62.7	39.7	20.1
Intestine	51.2	29.3	12.9	53.9	31.0	10.5
Muscle	46.8	34.8	21.5	48.1	23.2	11.6
Skin	48.7	25.7	12.3	60.4	28.7	17.5

Appendix 6. Concentration of T_3 (ng/g tissue) and tissue/plasma T3 concentration (T/P) values determined by RIA. Values corrected for 100% extraction.

	Fish 1 ♂		Fish 2 ♀		Fish 3 ♂		Fish 4 ♀	
Tissue	T_3	T/P	T_3	T/P	T_3	T/P	T_3	T/P
Liver	4.50	0.52	3.26	1.58	1.37	0.60	3.22	1.34
Kidney	6.50	0.76	2.30	1.12	0.95	0.42	3.60	1.50
Gill	3.16	0.37	1.20	0.58	0.69	0.30	1.92	0.80
Heart	4.31	0.50	2.58	1.25	1.36	0.60	1.30	0.54
Stomach	4.84	0.56	1.12	0.54	0.95	0.42	0.83	0.35
Intestine	3.37	0.39	2.57	1.25	1.32	0.58	1.71	0.71
Muscle	1.64	0.19	1.55	0.75	0.47	0.21	0.52	0.22
Skin	3.09	0.36	0.98	0.48	0.73	0.32	0.86	0.36
Plasma	8.58	-	2.06	-	2.28	-	2.40	-

Appendix 7. Tissue/plasma T_3 concentrations (T/P) ratios for unstressed, and stressed fish reaching isotopic equilibrium. T_3 concentrations are determined from chromatographs.

	T/P							
	unstressed					stressed		
Tissue	Fish 1 ♂	Fish 2 ♂	Fish 3 ♂	Fish 4 ♂	Fish 5 ♀	Fish 1[a] ♂	Fish 2[a] ♀	Fish 3 ♀
Liver	2.35	3.59	2.05	1.17	2.39	1.62	1.30	0.77
Kidney	2.73	2.49	3.03	-	2.30	3.60	2.33	0.90
Gill	0.63	0.58	0.32	0.57	0.47	0.51	0.23	0.20
Heart	1.41	1.08	0.46	0.57	0.42	0.77	0.47	0.60
Stomach	2.68	1.79	0.28	0.90	0.49	1.27	-	1.11
Intestine	7.86	4.22	0.97	4.73	1.16	2.78	1.33	1.45
Muscle	0.33	0.55	0.60	-	0.68	0.15	0.13	0.07
Skin	0.33	0.35	0.23	0.20	-	0.33	-	-

[a]pH = 4.8 + aluminum

[b]pH = 4.8

Appendix 8. Proportion of T_3 represented in chromatographs for unstressed and stressed fish reaching isotopic equilibrium. T_3 expressed as percentage of total cpm.

	% Chromatographic Radioactivity as $*T_3$							
	unstressed					stressed		
Tissue	Fish 1 ♂	Fish 2 ♂	Fish 3 ♂	Fish 4 ♂	Fish 5 ♀	Fish 1[a] ♂	Fish 2[a] ♀	Fish 3[b] ♀
Liver	22.9	52.1	51.1	23.8	53.7	28.5	31.9	47.7
Kidney	28.7	29.0	29.5	-	35.6	24.2	24.3	24.3
Gill	37.9	37.1	34.3	54.1	49.3	33.5	26.5	26.9
Heart	33.4	38.8	34.6	34.6	24.2	32.6	40.5	43.1
Stomach	42.0	46.4	21.7	36.6	36.0	36.9	-	45.5
Intestine	37.9	32.1	25.4	29.9	22.8	35.4	35.8	42.0
Muscle	64.0	75.3	75.5	-	70.0	41.7	38.4	32.6
Skin	31.6	32.9	30.2	32.3	36.9	44.4	-	-
Plasma	27.9	30.6	32.1	22.6	39.8	22.1	33.1	31.0

[a] pH = 4.8 + aluminum

[b] pH = 4.8

Appendix 9. T_3 concentration of tissue extracts of unstressed and stressed fish reaching isotopic equilibrium. Values are corrected for 100% extraction efficiency.

	T_3 (ng/g tissue)							
	unstressed					stressed		
Tissue	Fish 1 ♂	Fish 2 ♂	Fish 3 ♂	Fish 4 ♂	Fish 5 ♀	Fish 1[a] ♂	Fish 2[a] ♀	Fish 3[b] ♀
Liver	3.17	3.95	1.60	0.35	3.23	1.26	0.39	1.04
Kidney	3.69	2.74	2.36	-	3.11	2.81	0.70	1.22
Gill	0.85	0.64	0.25	0.17	0.63	0.40	0.07	0.27
Heart	1.90	1.19	0.36	0.17	0.57	0.60	0.14	0.81
Stomach	3.62	1.97	0.22	0.27	0.66	0.99	-	1.50
Intestine	10.61	4.64	0.76	1.42	1.57	2.17	0.40	1.96
Muscle	0.45	0.60	0.47	-	0.92	0.12	0.04	0.09
Skin	0.44	0.39	0.18	0.06	-	0.26	-	-
Plasma	1.35	1.10	0.78	0.30	1.35	0.78	0.30	1.35

[a] pH = 4.8 + aluminum

[b] pH = 4.8

Appendix 10. Linear equations for organ weights of rainbow trout. The analysis is based on body and organ weights of 48 fish ranging in weights from 171-702 g in the equations. y = organ weight in grams; x = body weight in grams.

Organ	Equation	r^2	p
Liver	y = 1.390 + 0.0073 x	0.44	<0.0001
Kidney	y = 0.735 + 0.0049 x	0.42	<0.0001
Gill	y = 1.029 + 0.0089 x	0.44	<0.005
Heart	y =-0.212 + 0.0027 x	0.31	<0.0001
Stomach	y = 0.977 + 0.0057 x	0.39	<0.0001
Intestine	y = 0.862 + 0.0022 x	0.08	n.s.