THE UNIVERSITY OF MANITOBA

SATURABLE 3,5,3' TRIIODO-L-THYRONINE BINDING SITES IN LIVER NUCLEI OF RAINBOW TROUT (*SALMO GAIRDNERI* RICHARDSON)

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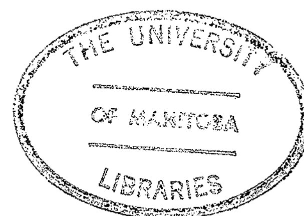

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BY

GLEN JOHN VAN DER KRAAK

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

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TO

MY WIFE AND PARENTS

ABSTRACT

Saturable binding of 3,5,3' triiodo-L-thyronine (T_3) was demonstrated in liver nuclei of rainbow trout using an in vivo isotope displacement technique.

The liver concentrated $^{125}I-T_3$ (T_3*) to a greater extent than other trout tissues. Maximal uptake of T_3* by liver nuclei occurred 8-16 hours after intraperitoneal injection. Isotopic equilibrium was assumed to occur 12 hours after injection. Saturable T_3-binding sites were demonstrated in the nuclear fraction by the reduced binding of T_3* in the presence of high levels of carrier T_3. A similar trend was not evident in the mitochondrial, microsomal or cytosol fractions.

Saturable binding sites were intranuclear and represented by a macromolecule extracted with 0.4M KCl. The macromolecule was identified as a heat-labile protein, probably non-histone in nature.

Saturable T_3-binding sites were characterized by high-affinity and low binding capacity. Equilibrium constants ranged from 0.9-1.2 X 10^8 kg liver/mole T_3 with binding capacities ranging from 0.43-0.62 X 10^{-9} mole T_3/kg liver. A 6-11-fold increase in plasma T_3 levels was required to saturate the nuclear sites. Approximately 50% of the sites were occupied at endogenous T_3 levels. When related to DNA content, starvation reduced the binding capacity by 24%, and the amount bound at endogenous T_3 levels by 37%.

The demonstration of saturable T_3-binding sites does not prove a role in hormone action, further study is required to determine the significance of these sites.

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CHAPTER 1 - INTRODUCTION

Thyroid hormones, L-thyroxine (T_4) and 3,5,3' triiodo-L-thyronine (T_3) affect growth, development and metabolic processes of vertebrates (Bernal and Refetoff, 1977). Definition of the mechanism of thyroid hormone action has been complicated by the diversity of hormonal effects and different potencies of T_4 and T_3. Suggested mechanisms of action have included thyroid hormone involvement with nuclear transcription, mitochondrial activation, stimulation of the sodium pump (Na-K ATP-ase) and increased transport through membranes (Bernal and Refetoff, 1977; Sterling, 1977). Recent attention has focused on a nuclear site of action with the demonstration of high-affinity low-capacity T_3-binding sites in nuclei from the liver and kidney of rats (Oppenheimer *et al.*, 1972). These binding sites could be saturated with T_3 since excess unlabelled T_3 displaced bound radioactive T_3. The nuclear T_3-binding sites are relatively specific, cross-reacting minimally with T_4. The affinity of these sites for T_3 was at least 20-fold greater than that for T_4, the less metabolically potent thyroid hormone (Oppenheimer *et al.*, 1974a).

Similar nuclear binding sites for T_3 have been described by *in vivo* and *in vitro* displacement techniques in a variety of tissues and cell types, including: GH_1 cells, a cultured rat pituitary cell line (Samuels and Tsai, 1973); rat hearts (Oppenheimer *et al.*, 1973); human lymphocytes (Tsai and Samuels, 1974) and human polymorphonuclear leukocytes (Woebber, 1977). Saturable T_3-binding nuclear sites have been characterized in non-mammalian tissues including the liver, lung and brain of chick embryos (Bellabarba and Lehoux, 1975) and bullfrog

tadpole liver (Kistler _et al._, 1975) and tail fins (Yoshizato _et al._, 1975).

Hypotheses implicating T3 action at the nuclear level involve the induction of a set of specific proteins that in turn activate chromatin (Surks _et al._, 1973; Spindler _et al._, 1975; Bernal _et al._, 1978b). Nuclear RNA synthesis (Tata and Widnell, 1966; DeGroot _et al._, 1977c; Dillman _et al._, 1978a), cytosol and nuclear protein synthesis (Oppenheimer _et al._, 1975 and 1977; Bernal _et al._, 1978a) are promoted in rat liver following T_3 administration. Nuclear T_3-binding sites have been characterized as chromatin-associated non-histone proteins (Surks _et al._, 1973; Samuels _et al._, 1974; MacLeod and Baxter, 1976). The latter finding is significant as this class of proteins is considered to regulate specific gene function.

The demonstration of specific T_3-binding sites has reinforced the theory implying T_3 as the active thyroid hormone with T_4 having a "prohormone" role (Ingbar and Braverman, 1975; Oppenheimer, 1975; Bernal and Refetoff, 1977). No studies have been published to date on the intracellular binding of T_3 in fish. However, indirect evidence suggests the importance of T_3. Eales (1977b) found as great a conversion of T_4 to T_3 in rainbow trout as has been estimated in mammals. In addition, trout with artificially increased T_4 levels are able to regulate and maintain relatively constant levels of T_3 (Brown _et al._, 1978).

The objective of this thesis has been to investigate intracellular T_3-binding in rainbow trout, _Salmo gairdneri_ Richardson, with particular reference to the nature of T_3-binding sites in the nucleus. Chapter 4 deals with the definition of a target tissue for studying T_3

uptake and the characterization of nuclear uptake of T_3, thereby allowing the nature of T_3-binding sites in the nucleus to be examined. Chapter 5 characterizes the nuclear components responsible for T_3-binding. In Chapter 6 the kinetic properties of nuclear T_3-binding sites have been studied in both fed and starved trout.

CHAPTER 2 - LITERATURE SURVEY

Several lines of evidence suggest that the intranuclear T_3-binding sites are loci initiating the biological effects of thyroid hormones. The ability of thyroid hormone analogues to displace T_3 from nuclear sites correlates closely with their reported thyromimetic activity (Oppenheimer et al., 1973; Samuels et al., 1973; Thomopolus et al., 1974; Koerner et al., 1975). The binding affinities of T_3 to nuclei of various rat tissues are similar, with different binding capacities which correlate with tissue responsiveness to administered hormone (Oppenheimer et al., 1974b). Rat pituitary, liver, kidney and heart, tissues responsive to administered thyroid hormones, have higher binding capacities than unresponsive tissues such as the spleen, testis and brain.

A relationship exists between nuclear occupancy and metabolic response (Oppenheimer et al., 1975 and 1977). Saturation of nuclear binding sites in rat liver is correlated with the maximal rate of synthesis of α-glycerophosphate dehydrogenase and malic enzyme. T_3 doses above the saturation level prolong the period of maximal enzyme synthesis without increasing the rate of enzyme synthesis. When nuclear sites are no longer saturated the rate of new enzyme appearance diminishes rapidly. A similar functional correlation between the occupancy of nuclear sites and tissue response was demonstrated by the increased synthesis of growth hormone by GH_1 cells in tissue culture (Samuels et al., 1976).

Abnormalities in human lymphocyte nuclear T_3-binding sites have been correlated with a syndrome of peripheral resistance to thyroid

hormone action (Bernal et al., 1975). In these cases retarded growth, stippled epiphyses and goiter were evident despite high levels of T_4 and T_3 in the plasma and tissues examined. T_3-binding sites in lymphocyte nuclei were absent or reduced tenfold and these sites also bound abnormally to chromatin. However, abnormal binding sites are not evident in all cases of peripheral resistance to thyroid hormones (DeGroot et al., 1978).

Additional indirect support for a functional role of T_3-binding sites became apparent as the intranuclear loci responsible for saturable binding were characterized. Saturable nuclear sites could be extracted in 0.4M KCl as macromolecular complexes, which were subsequently identified as non-histone proteins (Surks et al., 1973; DeGroot et al., 1974; Samuels et al., 1974). T_3 binding to isolated rat liver chromatin in vitro has been demonstrated by Thomopolus et al., 1974; Spindler et al., 1975; Yoshizato et al., 1977; and Bernal et al., 1978b. DeGroot et al. (1976a) demonstrated binding of T_3-macromolecular complexes extracted from rat liver to chromatin isolated from various rat tissues. They observed no clear relationship between extent of binding of such complexes and the T_3-responsiveness of tissues, and concluded that the controlling factor in the response of T_3 is the tissue concentration of the non-histone T_3-binding protein and its saturation. MacLeod and Baxter (1975 and 1976) demonstrated the binding of extracted nuclear T_3-binding complexes to DNA whereas free T_3 or T_3 bound to plasma proteins do not bind to DNA. Defer et al. (1975) found that T_3 bound to cytosol proteins does not bind to DNA. MacLeod and Baxter (1976) observed similar binding of T_3-protein complexes to rat

DNA, eucaryotic DNA from different species and procaryotic DNA. These studies suggest that the T_3-binding protein is a DNA binding protein and may, as a result, be a major determinant for receptor localization in the chromatin. Yoshizato et al. (1977) found hormone binding to chromatin to be inhibited by rifamicin, a eucaryotic RNA polymerase inhibitor. This suggests that the T_3-binding proteins may have properties in common with RNA polymerase and in some way RNA polymerase may be involved in the interaction of T_3 with chromatin. Latham et al. (1976) and Torressani and Anselmet (1978) have partially purified the nuclear T_3-binding protein. Their results suggest histones and DNA are not required for T_3-binding, while these components effect the stability of the T_3-binding protein.

The nuclear-initiation theory is based on the correlation of hormone-binding affinity with hormone activity. However several questions remain unanswered. Binding to nuclear sites may represent the first step in T_3 action, but the subsequent events and the coupling of metabolic responses are not known. Several mechanisms are theoretically possible (Bernal et al., 1978b). The nuclear sites may be receptors and hormone binding may induce conformational changes leading to receptor activation. Alternatively T_3-binding may transfer the receptor from inactive to active acceptors in the chromatin. These theories suggest that once the receptor is in the active form it would modify chromatin activity. The receptor could be a repressor with the role of T_3-binding as one of inactivation by affecting its catalytic properties or removing it from the specific acceptor sites in the chromatin (Bernal et al., 1978b). Contradictory reports regarding the nature of the receptor are

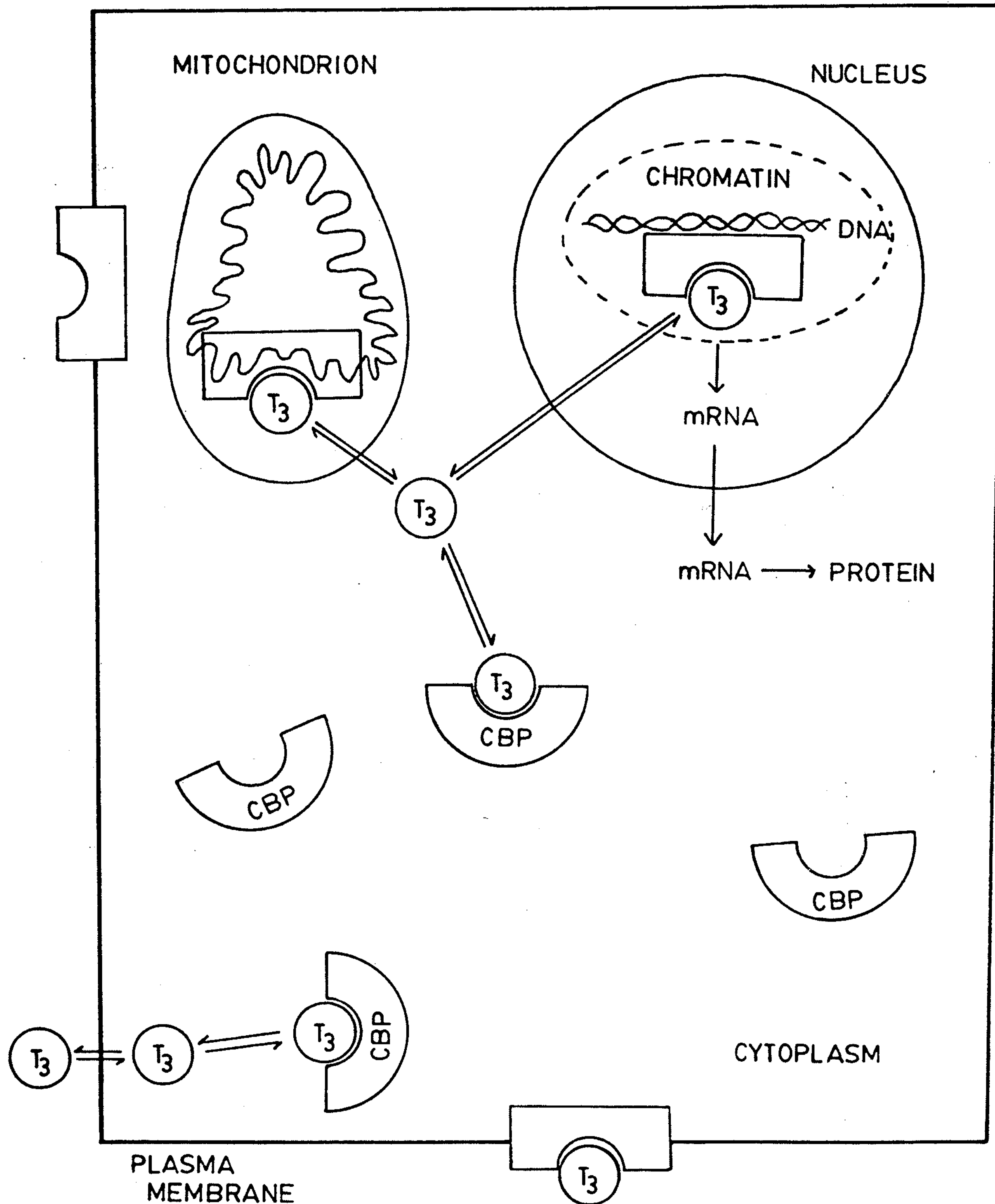
found in the literature. Samuels et al. (1976) using GH_1 cells in culture reported that T_3 induced a depletion of receptors when these cells were treated with hormone. It was proposed that the receptor in this case was a repressor as the induction of growth hormone synthesis by T_3 correlated better with receptor depletion than with hormone binding. However, in the rat, no difference in receptor binding was evident when hypothyroid, euthyroid or T_3-treated animals were compared (Spindler et al., 1975; DeGroot et al., 1976b; Bernal et al., 1978b). Attempts to demonstrate subtle conformational changes due to hormone binding have been successful to a limited extent. Silva et al. (1977) found occupied receptors bound more tightly to anionic exchange resins than empty receptors, possibly implicating that bound receptors could bind to receptor binding sites in the chromatin to a greater extent than free receptors. Bernal et al. (1978b) found that free receptors have a higher affinity for T_3 than receptors bound to the chromatin suggesting that binding to free receptors may increase the affinity of the receptor for the chromatin. However, no difference in binding to the chromatin was observed between occupied and unoccupied receptors; binding to chromatin was non-saturable in both cases (Silva et al., 1977; Bernal et al., 1978b). While specific acceptor sites for occupied receptors in the chromatin are probable, the methods to date are unable to distinguish these sites.

The theory of nuclear initiation of metabolic events discounts the greater than 85% of the intracellular T_3 that is extranuclear. T_3 increases the rate of mitochondrial RNA synthesis (Gadaleto et al., 1975; Barsano et al., 1977). Not all the effects of thyroid hormones

are easily interpreted in terms of a mechanism requiring protein synthesis. These include increased transport of amino acids and carbohydrates into cells, stimulation of the sodium pump and oxidative phosphorylation prior to the onset of increased protein synthesis (Bernal and Refetoff, 1977). Some actions may be explained by participation in tyrosine regulatory pathways (Dratman, 1974). High-affinity limited-capacity T_3-binding sites are not unique to the nucleus as sites with similar properties have been demonstrated in rat liver in association with inner mitochondrial membranes (Sterling and Milch, 1975; Sterling, 1977; Sterling et al., 1978) and plasma membranes (Pliam and Goldfine, 1977). The mitochondrial sites display traits similar to intranuclear T_3-binding proteins with respect to analogue displacement studies and concentrations of T_3-binding proteins in responsive and unresponsive tissues. Localization of high-affinity T_3-binding sites on inner mitochondrial membranes may provide an avenue for T_3 activation of oxidative phosphorylation. The significance of high-affinity T_3-binding sites in the plasma membrane is not known, but possible implications include regulation of amino acid, carbohydrate and its own uptake into responsive cells. With thyroid hormones having a ubiquitous distribution within cells multiple sites of action are possible. The final expression of thyroid hormone action may involve cooperation between both nuclear and extranuclear sites (Fig. 1).

While both thyroid and steroid hormones interact with the nucleus, the mechanisms of action demonstrate fundamental differences. A generalized scheme of steroid hormone action involves binding of the steroid to cytoplasmic receptors which then translocate to the nucleus

Figure 1. A model of T_3 interactions with recognized T_3-binding proteins in rat liver cells. CBP represents cytosol T_3-binding proteins. (Adapted in part from Sterling, 1977.)


MITOCHONDRION
NUCLEUS
CHROMATIN
DNA
T3
mRNA
T3
mRNA ⟶ PROTEIN
T3
CBP
CBP
CBP
T3
T3
T3
CBP
CYTOPLASM
PLASMA MEMBRANE
T3

and bind to chromatin stimulating RNA synthesis (Gorski and Gannon, 1976; Yamamoto and Alberts, 1977). Although specific thyroid hormone binding proteins have been demonstrated in the cytosol (Davis _et al._, 1974; Dillman _et al._, 1974; Sterling _et al._, 1974; Defer _et al._, 1975), cytosol proteins are not required to demonstrate specific binding to isolated nuclei _in vitro_ (Samuels _et al._, 1974; DeGroot and Torresani, 1975; Surks _et al._, 1975). Although T_3 binds to cytoplasmic sites it dissociates into the free form before entry into the nucleus. Cytosol T_3-binding proteins may act as intracellular transport proteins in an analogous manner to thyroid hormone-binding proteins in the plasma. The demonstration of steroid receptors depends on prior exposure to hormone. In contrast, the affinity and capacity of T_3-binding sites in liver nuclei were similar for euthyroid and thyroidectomized rats (Surks _et al._, 1975; DeGroot _et al._, 1976b; Bernal _et al._, 1978b) Unlike steroids, T_3 may interact with more than one high-affinity effector locus by binding to receptors in the nucleus, mitochondrion or plasma membrane.

CHAPTER 3 - MATERIALS AND METHODS

A. Fish Maintenance

One-and two-year-old rainbow trout, (British Columbia, Idaho and Montana stock), were obtained from the Federal Fish Hatchery, Balmoral, Manitoba. Trout stocks were held separately in 2.3 k-litre fiberglass tanks with flowing aerated, dechlorinated Winnipeg City water. Fish were fed a 1.0 to 2.0% (percentage wet body weight per day) ration of Ewos trout pellets (Astra Chemicals Ltd., Mississauga, Ontario). Iodide content of the food was 0.52 µgI/g dry weight (Hunt and Eales, 1979).

Experimental fish were transferred to uniform 125-litre fiberglass tanks for at least 7 days prior to use. Water temperatures varied from 11.2-12.5^{o} C. The photoperiod was 12 hr L : 12 hr D (light 0830-2030 hr). Experimental conditions are described in the protocol for individual experiments.

B. Injections

Subgroups of trout were transferred to plastic tanks containing 25 litres of isothermal aerated water. Prior to injection or blood sampling individual trout were anesthetized by immersion in tricaine methane sulfonate (MS222, Syndell Laboratories Ltd., Vancouver; 0.08g/litre).

Isotopic T_3* (^{125}I-T_3, labelled in the phenolic ring, initial Specific Activity 550 mCi/mg; Industrial Nuclear Co., St. Louis) and nonisotopic T_3(^{127}I-T_3, anhydrous sodium salt, Sigma) were dissolved in 50% propylene glycol. Hormones were injected in a total volume of 20 µl from a 1-ml tuberculin syringe (needle size 30G, ½") adapted to a

repeating dispenser (PB-600-1, Hamilton Company, Reno, Nevada). In Experiment I CHAPTER 4 a cardiac injection route was used. Injection was at a point opposite the origin of the pectoral fins along the midventral line with the needle pointed downward. Routinely, separate intraperitoneal injections of T_3* and carrier T_3 were made anterior to the pelvic fins. At the time of injection aliquots of injected T_3* were dispensed to determine the injected T_3* dose which was also corrected for the $^{125}I^-$ contamination (see Section I).

C. Blood and Tissue Sampling

Blood was removed from the caudal vessels with a preheparinized 3-ml tuberculin syringe (needle size 25G, 5/8 or 7/8"). Blood was expelled into 1.5-ml plastic centrifuge tubes and held on ice until centrifugation at 15,000g for 3 min (International Centrifuge Model MB). The separated plasma was stored in 2-ml plastic beakers covered with Parafilm at -20° C. Fish were killed by concussion, blotted dry and weighed to the nearest 0.1g.

Whole organs or tissues were removed from freshly-killed trout. The whole organ or a weighed portion was placed in a counting tube and made up to a volume of 4 ml with 0.1N NaOH for counting purposes. When the whole intestine was utilized, the tissue was divided between several tubes each adjusted to 4.0 ml with 2N NaOH and allowed to partially digest prior to counting. Radioactivity was expressed as % injected T_3* dose per organ or % injected T_3* dose/g tissue. Radioactivity in blood cells was calculated indirectly from the hematocrit (HCR) and the radioactivity per g of whole blood and plasma:

$$\text{Radioactivity in blood cells (cpm)} = \frac{\text{Whole blood(cpm)} - \text{(1-HCR)plasma(cpm)}}{\text{HCR}}$$

Radioactivity was expressed as % injected T_3* dose/g blood cells.

D. Isolation of Subcellular Fractions

After dissection fresh livers were stored on ice and a weighed portion used to isolate subcellular components. All subsequent steps were performed at 0-4°C.

Two buffers, A and B, were used. Both contained 3mM $MgCl_2$, 1mM dithiothreitol, 1mM spermidine and 10mM tris, pH 7.7. Buffer A contained 0.32M sucrose; Buffer B contained 2.3M sucrose. Spermidine prevented the formation of viscous aggregates that could not be properly sedimented by centrifugation (Tarr and Gardner, 1970).

An individual liver was homogenized in four volumes of Buffer A by four strokes at 700 rpm of a tight-fitting motorized pestle (Tri-R Stir-R, Tri-R Instruments Inc., New York). The homogenate was transferred to a 13-ml capacity Autoclear polycarbonate centrifuge tube (Fisher). The homogenizer chamber was flushed with four volumes of Buffer A and combined with the homogenate. Subsequent centrifugation steps for the isolation of the nuclear fraction were performed using a refrigerated centrifuge (Model B-20, International Equipment Co., Needham Hts., Mass.). The homogenate was centrifuged for 10 min at 700g to obtain a crude nuclear pellet. The supernatant was decanted and saved in one experiment (Experiment III Chapter 4). Eight volumes of Buffer B were added to the crude nuclear pellet, which was gently rehomogenized with a loose-fitting hand-held scintered glass pestle. The suspension was centrifuged at 35,000g for 1 hr. A floating crude nuclear residue was removed with a spatula and the supernatant decanted leaving the nuclei as a greyish-white pellet. Purified nuclei, as

viewed by interference microscopy, were intact and relatively free of cellular debris and whole-cell contamination.

Radioactivity was generally expressed as % injected T_3* dose in nuclear fraction/g liver or N/L, the proportion of liver radioactivity in the nuclear fraction. Alternative ways of expressing nuclear radioactivity are described in the appropriate experiments.

Mitochondrial, microsomal and cytosol fractions were obtained by serial differential centrifugation of the 700g supernatant in a Beckman Model L3-50 Ultracentrifuge; ti-50 rotor. Centrifugation for 20 min at 15,000g resulted in a crude mitochondrial pellet. The decanted supernatant spun at 100,000g for 1 hr resulting in a microsomal pellet. The supernatant was designated as cytosol. Radioactivity in individual mitochondria, microsomal and cytosol fractions was expressed as the % of liver radioactivity per fraction.

E. Sucrose Buffer and Triton X-100 Extraction of Nuclear T_3*

Sucrose buffer C (0.32M sucrose 3mM $MgCl_2$ pH7.6) was added to the purified nuclear pellet (5.0ml/g liver) and the pellet gently resuspended. A sample was removed to determine total nuclear binding and separate 1 ml samples were transferred to 1.5-ml polystyrene centrifuge tubes which were held on ice for 15 min and then centrifuged at 15,000g (International Centrifuge Model MB) for 3 min. The supernatant containing extracted T_3* was saved. The nuclear pellet was resuspended in 1.0 ml of sucrose buffer C containing 0.2% Triton X-100, the extraction repeated and the supernatant and pellet saved. The radioactivity in each fraction was counted and expressed as a % of radioactivity in all fractions combined.

F. Characterization of Nuclear-Bound T_3*

Cold buffer (0.4M KCl, 0.01M Tris pH7.6) was added to the purified nuclear pellet (1.5ml/g liver) and the pellet resuspended by 4 strokes at 700 rpm of a motorized pestle. More buffer (0.5 ml/g liver) was added, the tube vortexed for 5 seconds and a sample removed to determine total nuclear T_3*. The remaining suspension was transferred to a 1.5-ml polystyrene centrifuge tube and placed on ice for 1 hr Following centrifugation for 3 min at 15,000g (International Centrifuge Model MB) the supernatant containing the KCl extract was aspirated and collected. The residual pellet representing unextracted T_3* and the centrifuge tube were counted. Binding of T_3* to the centrifuge tube was negligible ($<0.3\%$). The extraction efficiency of the KCl treatment was determined by comparing the residual pellet with the total nuclear T_3*.

$$\%\ \text{extracted} = 100\left(1 - \frac{\text{pellet cpm}}{\text{homogenate cpm}}\right)$$

Sephadex columns, prepared as described for RIA (Brown and Eales, 1977; see Section H), were equilibrated at pH 7.6 by elution with 10 ml of KCl buffer. The columns were capped at the bottom, 0.2ml of KCl extract applied and gently swirled. The cap was removed and the KCl extract allowed to drain into the column. The following sequential elutions were made; each eluate was collected in a separate counting tube. Two ml of KCl buffer removed macromolecular-bound T_3* in the void volume (as determined by Dextran Blue). The next 3 ml of KCl buffer removed the inorganic iodide present in the KCl extract. A further 2 ml of KCl buffer was added to check the efficiency of iodide

removal. This volume represents the gap between the iodide and free T_3 fractions. Free T_3 was removed with two 4 ml washes with human plasma in KCl buffer (1:9) (Appendix 1). The average recovery of added radioactivity from nuclear T_3* KCl extracts was 96% (range 90-116%). The radioactivity in each fraction was counted and expressed as a % of radioactivity in all fractions combined.

G. Stability of Macromolecular-Bound T_3*

The effects of temperature and various enzymes on the stability of macromolecular T_3* complexes were assessed using a common pool of KCl extract. Deoxyribonuclease 1 B grade (DNase), ribonuclease 5A (RNase) and α-chymotrypsin B grade were obtained from Calbiochem. Trypsin was obtained from Fisher and Pronase Type VI was obtained from Sigma. Experimental tubes were made in triplicate containing 0.2 ml of KCl extract and 0.1 ml of KCl buffer or enzyme in KCl buffer (0.5 mg/ml). Samples were incubated for 1 hr at 0, 25 or 37°C for temperature treatments and 0°C for enzyme treatments. An unincubated group served as a control for the temperature study and the 0°C tubes from the temperature study served as a control for the enzyme study. Following incubation 0.2 ml was removed and the proportions of radioactivity in the macromolecular, iodide and T_3 fractions determined (see Section F).

A separate experiment was conducted to assess the binding of free T_3* to enzyme preparations. Protocol paralleled previous enzyme treatments except that T_3* in KCl buffer was substituted for the KCl extract. In these cases less than 0.5% of the T_3* eluted in the void volume. This indicates that macromolecular-bound T_3* in KCl extracts following enzyme treatments was not a consequence of free T_3* binding to the enzymes.

H. T_4 and T_3 Measurement

Plasma T_4 and T_3 were measured by radioimmunoassay (RIA) (Brown and Eales, 1977). The assay was conducted on miniature columns (5-ml syringe barrel) prepared from 0.45g of G-25 (fine) Sephadex. T_4 and T_3 antibodies from lyophylized rabbit antisera to T_4 and T_3 human serum albumin respectively were purchased from K&T Biological Services Ltd., Edmonton. The T_3 antiserum was diluted 1:22,000 and the T_4 antiserum was diluted 1:5,000 with barbital buffer (sodium barbital, 75mM pH8.6). The suppliers specify that T_4 and T_3 antibodies have low cross reactivity with each other and other iodothyronines.

Standard stock solutions of T_3 and T_4 were prepared by dissolving the anhydrous sodium salt of T_3 (Sigma) or T_4 tablets (Eltroxin, sodium L-thyroxine pentahydrate) in 0.1N NaOH. Working standards of 0-800 ng % were prepared by dilution with 0.1N NaOH.

T_4* (^{125}I-T_4, phenolically labelled, initial Specific Activity 750 mCi/mg; Industrial Nuclear Co., St. Louis) and T_3* were diluted with 0.1N NaOH to provide separate stock solutions. A 0.1 ml aliquot of stock solution contained 5,000-7,000 cpm in a gamma well-counter of about 50% efficiency.

Columns and reagents were equilibrated to room temperature before use. Columns were drained and the bottoms capped prior to the addition of 0.1 ml of T_3 or T_4 standard or 0.1 ml plasma. Standards and plasma were analyzed in duplicate. A 0.1 ml aliquot of T_3* or T_4* was added to each column and separate counting tubes for determination of the added dose. The columns were swirled and allowed to drain. When plasma enters the column equilibrated with 0.1N NaOH the T_3 and T_4

dissociate from the plasma proteins and bind to the Sephadex. Elution with 3.0 ml of barbital buffer for T_3RIA or 4.0 ml of barbital buffer for T_4RIA removes iodide and plasma proteins. Routinely this eluate was collected from four randomly chosen columns to determine radioiodide contamination of the T_3* or T_4*.

Counting tubes were positioned under the columns and in sequence 1.0 ml of T3 or T4 antibody was added to the columns. The columns were covered and incubated for 90 min. In sequence 2.0 ml of barbital buffer (T_3RIA) or 3.0 ml of barbital buffer (T_4RIA) were then added and the eluate containing antibody bound hormone collected. Tubes containing the iodide fraction, antibody bound fraction and the added radioactivity were adjusted with 0.1N NaOH prior to counting. T_3 and T_4 standard curves were formed by plotting antibody bound cpm on the ordinate versus hormone concentration. Plasma hormone concentrations were determined by interpolation.

Columns were regenerated by elution with 10 ml of deionized water followed by 8 ml of human plasma in barbital buffer 1:9, 10 ml of deionized water, and 8 ml of 0.1N NaOH. Columns were stored capped with 1-2 ml of 0.1N NaOH above the column surface.

I. Separation of Plasma ^{125}Iodide from T_3*

Plasma radioactivity was separated into ^{125}Iodide and T_3* using a modification of the method described by Eales (1977a). Sephadex columns identical to those used for RIA were adjusted to pH 13 with 0.1N NaOH and the bottoms capped. Plasma (0.05 or routinely 0.1 ml) and 0.1 ml of 0.1N NaOH were added to the top of the column and gently swirled. A counting tube was positioned under the column, the bottom

cap removed and the sample drained onto the column. The column was eluted with 2.8 ml of barbital buffer to collect the ^{125}Iodide fraction. T_3* was removed in two washes with 4.0 ml of human plasma in barbital buffer (1:9) and the eluates collected separately. The plasma iodide contamination was expressed as a percentage of the total eluted radioactivity. Human plasma removed all of the column residual radioactivity. Iodide contamination varied by less than 1% between duplicate plasma samples. Plasma T_3* corrected for iodide contamination was expressed as the % of the injected T_3* dose per g of plasma. The ^{125}Iodide contamination of the injected T_3* dose was determined in a similar fashion.

J. DNA Measurement

Liver homogenate (0.05g/ml) or purified nuclei (0.2g original liver wt./ml) in Buffer A were frozen up to two weeks before analysis. Freezing did not effect DNA determination.

DNA was extracted using a modification of the technique described by Widnell and Tata (1964). One ml aliquots of thawed preparation were mixed with 8 ml of 0.2N $HClO_4$ and placed on ice for 15 min. The samples were centrifuged at 10,000g for 10 min at 0-4°C. The supernatant was discarded and the above procedure repeated using the residual pellet. Four ml of 0.5N $HClO_4$ was added to the pellet followed by heating at 75°C for 15 min in an oven or water bath. The sample was cooled on ice and centrifuged as above. The supernatant containing hydrolysed DNA was collected and the high-temperature extraction repeated with 3.5 ml of 0.5N $HClO_4$. Supernatants were pooled for each individual tube, adjusted to 8 ml and a 1 ml aliquot used for DNA determination.

DNA was estimated colorimetrically by the diphenylamine reaction (Burton, 1956) using calf-thymus DNA (Sigma) as a standard. Diphenylamine reagent (1.5g diphenylamine, 100 ml glacial acetic acid, 1.5 ml concentrated sulphuric acid and 8.12 mg aqueous acetaldehyde) was prepared fresh. A solution of 0.4 mg DNA/ml 0.005 M NaOH was mixed with an equal volume of 1N $HClO_4$. The solution was heated twice at 75°C for 15 min and used as a DNA stock solution. One ml DNA standards were prepared by mixing known amounts of DNA stock solution with 0.5N $HClO_4$. Two ml of diphenylamine reagent was added to 1 ml of test solution and colour was developed by incubating at 30°C for 20 hr in a water bath. Absorbance at 595 mμ was measured against the blank in a Baush and Lomb 'Spectronic 20' spectrophotometer. Absorbance (ordinate) was plotted against DNA concentration. A linear relationship was evident and unknowns were determined from the regression equation.

K. Radiation Counting

Counting was done in duplicate in a Nuclear Chicago Automatic Gamma System containing a 5.08 cm (DS202) NaI crystal. Samples were counted for a minimum of 10 min or 10,000 counts.

L. Statistical Analysis

Comparisons between two experimental groups were made using Students t-test when a two-tailed F-test showed that the variances were homogeneous. If heterogeneous variances were evident, a Behrens-Fisher's test was used. Bartlett's X^2 test was used to test homogeneity of variance when more than two groups were considered. One-way analysis of variance was used to test if means differed between groups. The statistical techniques used are described in Steele and Torrie (1960) and Snedocor and Cochran (1971).

CHAPTER 4 - DEMONSTRATION OF SATURABLE T_3-BINDING SITES

A. Objective

In mammals, physiologically relevant high-affinity intracellular T_3-binding sites are present in low numbers. These sites can be demonstrated in the nuclear fraction of T_3-responsive tissues by the reduced binding of T_3* in the presence of high levels of carrier T_3. In trout and other fish limited data are available to indicate tissues that are T_3-responsive. Consequently, it was necessary to survey the various trout tissues to determine which of them were prominent in accumulating T_3 (Experiment 1). It was felt that such tissues would be the most likely to demonstrate saturable binding of T_3. In this experiment plasma ^{125}I-levels were also monitored, to confirm reports of negligible deiodination of injected T_3* in trout (Eales, 1977b; Higgs and Eales, 1977). Negligible T_3 degradation is a premise for subsequent kinetic studies. Secondly, the time-course of T_3* uptake into the nuclear fraction was determined (Experiment II). The time of maximal nuclear uptake was to be used as the sampling time in all subsequent experiments. Finally, the presence of saturable T_3*-binding to the nuclear fraction and other subcellular fractions was determined by varying the dose of carrier T_3 (Experiment III).

B. Protocol

I. Tissue uptake of T_3*

Montana trout were maintained on a 1.8% food ration for 12 days and then starved 3 days prior to cardiac injection of T_3* (0.30μCi, iodide contamination 4.0%). Subgroups of 8 trout (mean wt. 118.7 g, SEM 2.5g) were bled and killed 4, 6, 9 and 12 hr after injection and radioactivity in various tissues assessed.

II. Time-course of liver nuclear T_3* uptake

Two groups of Idaho trout fed a 1.8% ration for 7 days were starved 3 days prior to intraperitoneal T_3* injection (Group A, 0.21 μCi, iodide contamination 2.5%; Group B, 0.26μCi, iodide contamination 2.7%). Fish from Group A (n=26, mean wt. 47.0g, SEM 1.7g) were killed at intervals from 0.5-24 hr whereas fish in Group B (n=29, mean wt. 47.7g, SEM 1.6g) were killed at intervals from 4-24 hr. Radioactivity in the plasma, liver and liver nuclear fraction was determined for each fish.

III. T_3* uptake into liver subcellular fractions

Idaho trout were divided into 2 groups of 12 fish (A, mean wt. 73.5g, SEM 2.8g; B, mean wt. 69.3g, SEM 2.0g). Fish were maintained on a 1.7% food ration for 10 days and then starved 3 days prior to intraperitoneal injections of T_3* (0.30 μCi, iodide contamination 1.9%) and 0 ng T_3 (Group A) or T_3* and 1250 ng T_3 (Group B). Fish were bled and killed 12 hr after injection. The uptake of T_3* into various liver subcellular fractions was compared between the two groups. Crude nuclear pellets were counted from 4 fish randomly chosen from each group; the remaining 8 fish provided nuclear pellets. Mitochondrial, microsomal and cytosol fractions were obtained from all fish.

C. Results

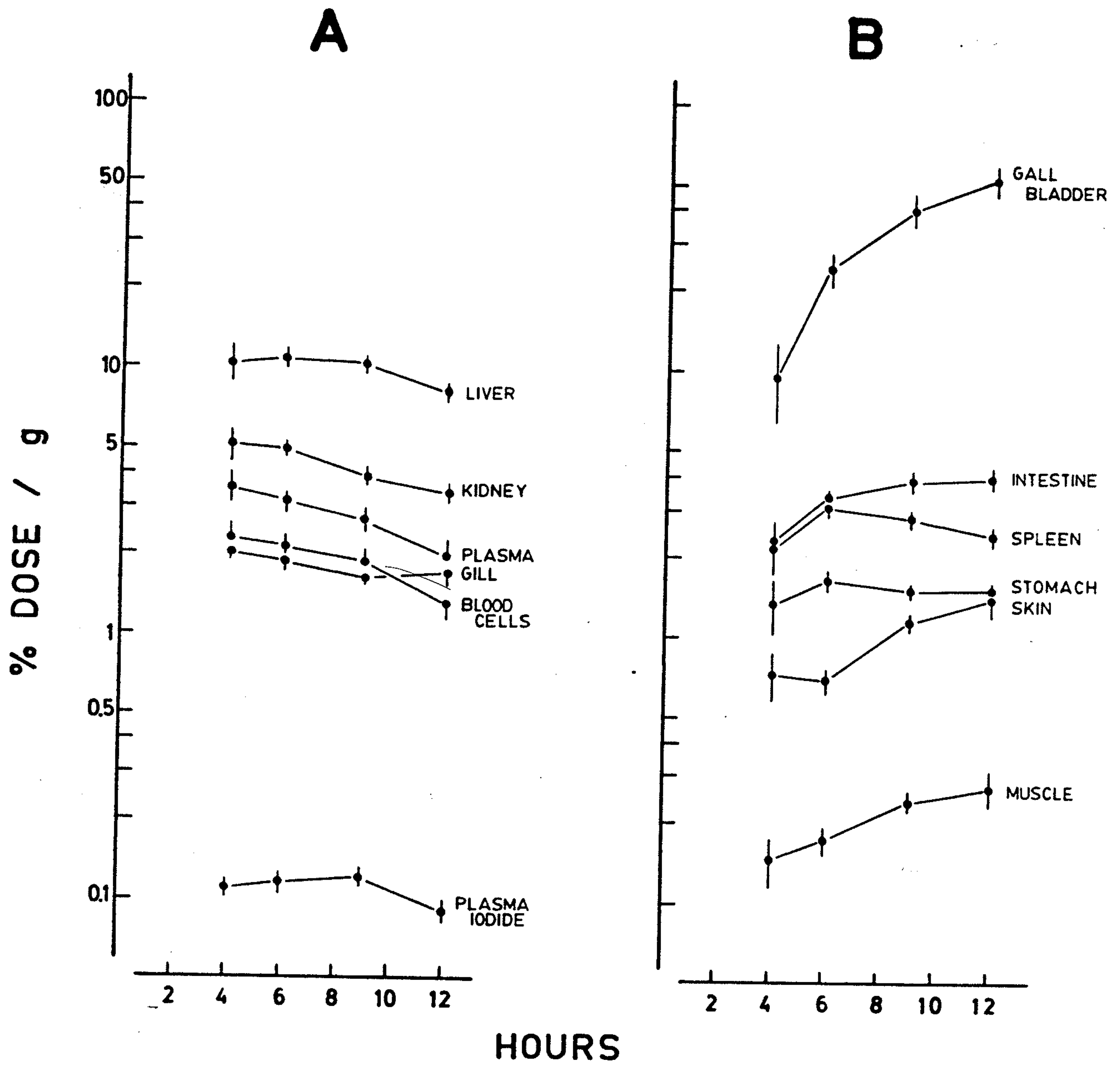
I. Tissue uptake of T_3*

Figure 2 illustrates the distribution of radioactivity in the plasma and various tissues following cardiac T_3* injection. Tissues were categorized as rapidly - or slowly - equilibrating. Rapidly-equilibrating tissues generally followed the distribution of T_3* in the plasma. Slowly-equilibrating tissues were characterized by a period of

Figure 2. Uptake of radioactivity into tissues of trout injected into the heart region with a tracer dose of T_3*. Each point represents a mean ($\pm$ 2 SEM) of determinations from 8 fish. SEM are omitted in several places to avoid congestion.

A. Total radioactivity in rapidly-equilibrating tissues and plasma iodide fractions.

B. Total radioactivity in slowly-equilibrating tissues. (Fish sampled at 0.5, 1 and 2 hr after injection were not included. In these cases, tissue radioactivities were highly variable conforming to differential rates of uptake into the plasma.)


A
B
100
50
10
5
1
0.5
0.1
% DOSE / g
LIVER
KIDNEY
PLASMA
GILL
BLOOD CELLS
PLASMA IODIDE
GALL BLADDER
INTESTINE
SPLEEN
STOMACH
SKIN
MUSCLE
2
4
6
8
10
12
HOURS

increased uptake despite decreasing plasma T_3*.

Low levels of plasma radioiodide suggest that the injected T_3* is not deiodinated. This is of major importance in the evaluation of T_3* kinetics *in vivo*, as tissue radioactivity can be assumed to represent labelled T_3.

Estimates of tissue T_3* prior to 4 hr were highly variable with a distinct uptake phase (see Legend Fig. 2). This was unexpected as injection directly into the blood stream should not result in a phase of T_3* uptake into the plasma. As a result, later studies employed an intraperitoneal injection site which provided a slower, but more uniform plasma T_3* uptake. Studies utilizing the intraperitoneal route provided data with a similar pattern of T_3* accumulation in the tissues studied.

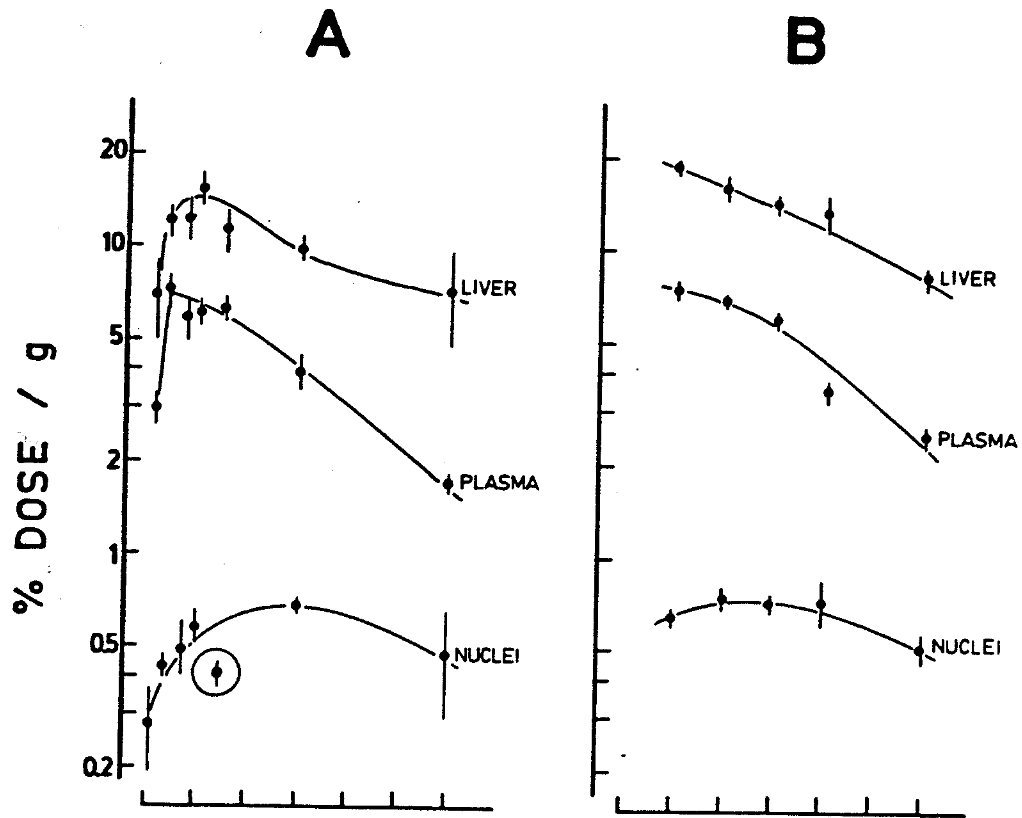
II. Time-course of liver nuclear T_3* uptake

Subsequent kinetic analyses necessitate measurement of nuclear T_3*-binding when it is maximal and also when the liver and plasma are acting as a single kinetic compartment. At this time isotopic equilibrium may be assumed. Twelve hr after T_3* injection was chosen. This represents the midpoint of the nuclear uptake plateau which extended from 8-16 hr (Fig. 3). Sampling at 12 hr conforms to the period of a constant L/P ratio, and thus when the liver and plasma are functioning as a single kinetic compartment. This was also the time at which N/L was maximal, during the period L/P was constant.

III. T_3* uptake into liver subcellular fractions

The percentages of liver T_3* associated with the crude and purified nuclear fractions were reduced by addition of carrier T_3 (Table 1). Similar trends were not evident in the mitochondrial,

Figure 3. Time-course of T_3* uptake into the liver, plasma and liver nuclei in two separate experiments, A and B, with corresponding L / P and N / L ratios. Values reported are the mean ($\pm$ 2 SEM) of 3-4 fish in experiment A or 5-6 fish in experiment B.


A
B
% DOSE / g
20
10
5
2
1
0.5
0.2
LIVER
PLASMA
NUCLEI
LIVER
PLASMA
NUCLEI

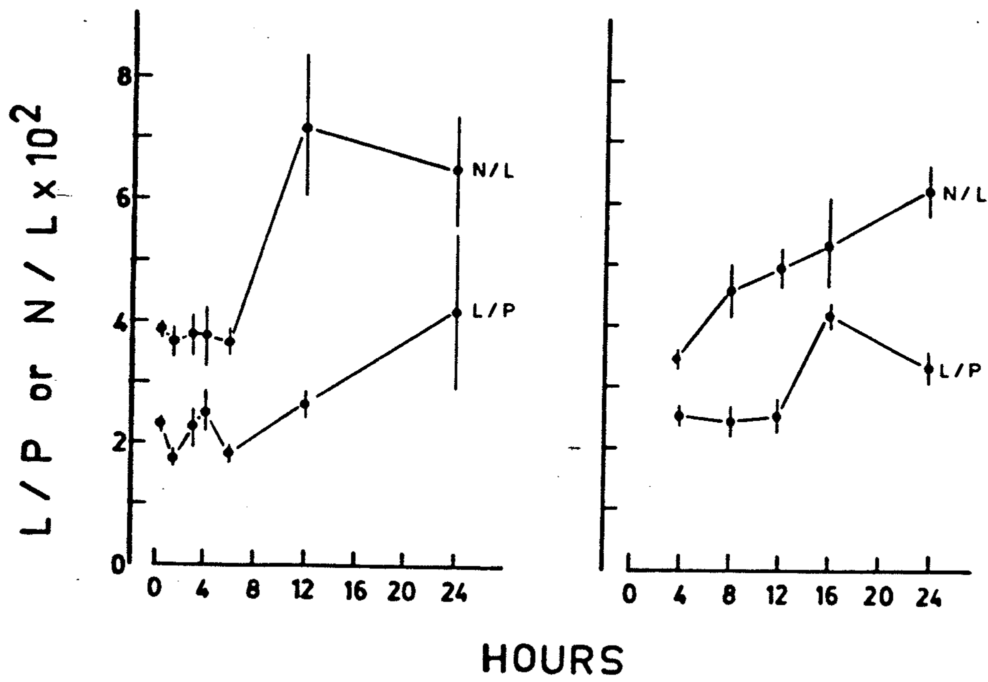

L / P or N / L x 10^2
8
6
4
2
0
N / L
L / P
N / L
L / P
0
4
8
12
16
20
24
0
4
8
12
16
20
24
HOURS

Table 1.

Percent of liver radioactivity associated with various subcellular fractions in trout killed 12 hr after injection of T_3* and either 0 or 1250 ng T_3. Statistical comparisons were made using a two-tailed t-test (not significant when P>0.05).

Fraction	0 ng			1250 ng		
	n	$\bar{x}$(%)	SD	n	$\bar{x}$(%)	SD
Crude nuclei	4	21.9	2.4	4	14.6^{+}	3.1
Purified nuclei	8	5.4	1.1	8	1.9^{+}	0.3
Mitochondria	12	22.9	2.1	11	25.3	5.5
Microsomal	12	14.7	2.7	11	12.8	3.7
Cytosol	11	31.4	4.1	11	37.7^{+}	5.0
Recovery^{++}		90.9			90.4	

$^{+}$ Significantly different from 0 ng group (P<0.01).

$^{++}$ Sum of crude nuclear, mitochondrial, microsomal and cytosol fractions.

microsomal or cytosol fractions. Thus the nuclear fraction was characterized by saturable uptake whereas the other subcellular fractions displayed non-saturable uptake. Furthermore, the reduced percentage of T_3* in the nuclear fraction with high levels of carrier T_3 occurred without altering the total liver T_3* content of the L/P ratio (Table 2). This indicates that carrier T_3 had not altered the kinetics of T_3* transfer between plasma and liver; and that T_3* was displaced by carrier T_3 from a limited number of nuclear sites, to sites in other fractions in the cell. In support of this, the reduction of nuclear T_3* was accompanied by increased mitochondrial and cytosol T_3* (Table 1).

D. Discussion

The liver was shown to contain saturable T_3-binding sites in the nuclear fraction. These sites were not unlike saturable T_3-binding sites described in rat liver nuclei (Oppenheimer et al., 1972 and 1974a; DeGroot and Strausser, 1974).

The patterns of radioactive uptake into the gall bladder, intestine, liver and plasma were comparable to those reported for brook trout (Eales et al., 1971; Higgs and Eales, 1977). High levels of radioactivity in the gall bladder and intestine would reflect in part the biliary excretion pathway known to feature prominently in T_3 clearance from the plasma (Sinclair and Eales, 1972; Eales and Collicutt, 1974) and would not necessarily represent tissue uptake. Consequently, on the basis of its rapid equilibration with plasma, its high uptake of radioactivity and its large size, the liver was chosen for the focus of subsequent study of nuclear binding.

Table 2.

Comparison of body weight and tissue uptake of T_3* in trout receiving 0 or 1250 ng T_3. Mean values ($\bar{x}$) and standard deviations (SD) are reported for 12 fish per group. Statistical comparisons were made using a two-tailed t-test (not significant, when $P>0.05$).

	0 ng		1250 ng	
	$\bar{x}$	SD	$\bar{x}$	SD
Weight (g)	73.5	9.6	69.3	6.9
Plasma (% dose/g)	6.6	1.2	7.9^{+}	1.5
Liver (%dose/g)	12.2	2.4	13.7	3.0
Gall bladder (% dose)	8.5	3.0	7.4	1.8
Liver / plasma^{++}	1.9	0.3	1.7	0.4

$^{+}$ Significantly different from 0 ng group ($P<0.02$).

$^{++}$ Liver / plasma = $\frac{T_3\text{* cpm/g liver}}{T_3\text{* cpm/g plasma}}$.

The pattern of T_3* uptake into the liver nuclear fraction of trout was different from that reported for the rat. In the rat maximal nuclear uptake was defined by a distinct peak following maximal liver uptake, and evident 0.5 hr (Oppenheimer et al., 1974a) or 2 hr (DeGroot and Strausser, 1974) after intravenous injection. The marked difference in the time course of nuclear uptake between the trout and rat relates to major differences in T_3 metabolism in these two vertebrate classes. Of major significance is the slow plasma clearance of T_3 in trout, due in part to the negligible degradation by deiodination and a reduced tendency for T_3 to leave the plasma and enter the tissues. Some of these differences in turn may relate to the low temperature (12°C) at which T_3 uptake was studied in trout.

The pattern of subcellular uptake in the present study (Table 1) was similar to that reported for rat liver (Oppenheimer et al., 1972) and GH_1 cells grown in tissue culture (Samuels and Tsai, 1973). However, one cannot rule out the possibility of saturable sites in other subcellular fractions as their presence may be masked by high levels of non-saturable binding. The demonstration of saturable T_3-binding sites in rat liver mitochondria (Sterling and Milch, 1975) and plasma membranes (Pliam and Goldfine, 1977) required highly purified preparations.

Saturable nuclear sites were demonstrated by the reduced binding of T_3* in the presence of high carrier T_3. The amount of saturable uptake can be determined by subtracting the percentages of liver radioactivity in the nuclear fraction of trout labelled in the absence or presence of high carrier T_3. This calculation assumes that

the high carrier T_3 dose represents the lower limit of nuclear T_3* uptake and was sufficient to fill the saturable nuclear sites with carrier. This seems to be the case, as higher doses of carrier T_3 (2,500 and 5,000 ng T_3, data not shown) did not further depress the percentage of nuclear radioactivity. So at high carrier T_3 doses the nucleus was non-saturable, as was the case for the other subcellular fractions, suggesting that carrier T_3 was bound to all saturable sites. In the present study, saturable binding represents 7.3% of the liver radioactivity when crude nuclear pellets are assayed and declined to 3.5% in purified nuclear pellets. This lower level of saturable binding can be attributed to a loss of nuclei during formation of the purified nuclear pellet. Based on an average recovery of 59% of the liver DNA in the purified nuclear pellet and correcting for DNA losses, the difference in saturable binding between crude and purified nuclear pellets was less than 20%. The 3.5% value due to saturable uptake in trout liver was considerably lower than comparable estimates for rat liver which averaged 6-11% (Oppenheimer _et al_., 1972, 1974a and 1974b; DeGroot and Strausser, 1974).

Saturable liver uptake should be evident by a reduced L/P ratio at high carrier T_3 levels. However, in the trout (Table 2) and rat this is not the case, as the level of saturable binding is low. Had 50% of the intracellular T_3* bound to saturable sites as in rat pituitaries (Schadlow _et al_., 1972; Oppenheimer _et al_., 1974b), saturable uptake would be evident in unfractionated liver by a significant decline of the L/P ratio.

CHAPTER 5 - LOCALIZATION AND CHARACTERIZATION OF SATURABLE NUCLEAR T_3-BINDING SITES

A. Objective

In Chapter 4 it was shown that T_3 binds to saturable nuclear sites. The main objective of this chapter has been to determine the nuclear component responsible for saturable T_3-binding. The first step was to distinguish T_3 bound to the exterior of the nucleus from that bound to intranuclear sites. This was done by comparing T_3 binding between nuclei with the outer membrane intact and nuclei with the outer membrane removed by Triton X-100 (Experiment 1). The second step was to ascertain if intranuclear binding sites were represented in the macromolecular fraction eluting from G-25 Sephadex columns (Experiment II). The final step was to characterize the stability of macromolecular T_3 complexes following treatment at different temperatures and with various enzymes (Experiment III).

B. Protocol

I. Effects of Triton X-100 extraction on nuclear-bound T_3*

A group of 8 Idaho trout (mean wt. 120.8g, SEM 8.3g) were maintained on a 1.7% food ration for 4 days and then starved 3 days prior to injection. Each trout received both an intraperitoneal injection of T_3* (1.1µCi, iodide contamination 3.3%) and either 0 or 10,000 ng T_3. Purified nuclear pellets prepared 12 hr after injection were subjected to extractions with sucrose buffer and sucrose buffer containing Triton X-100.

II. Characterization of nuclear-bound T_3* in KCl extracts

Three groups of 8 Idaho trout were maintained on a 1.8% food ration for at least 6 days and then starved 3 days prior to injection

(Group A, mean wt. 123.5g, SEM 7.0g; Group B, mean wt. 140.1g, SEM 10.0g; Group C, mean wt. 145.8g, SEM 6.6g). Trout were injected intraperitoneally with T_3* (1.0 µCi, iodide contamination 4.0%) and either 0 ng T_3 (Group B and 4 fish in Group A) or 10,000 ng T_3 (Group C and 4 fish in Group A). Purified nuclear pellets prepared 12 hr after injection were extracted with KCl and macromolecular-bound T_3* assessed by filtration through G-25 Sephadex columns.

III. Stability of macromolecular T_3* complexes

Portions of the KCl extracts prepared from fish in Group B of the previous experiment were combined to form a common pool of extract. This pool was used to assess the stability of macromolecular T_3* complexes following treatment at different temperatures and with various enzymes.

C. Results

I. Effects of Triton X-100 extraction on nuclear-bound T_3*

Saturable nuclear T_3*-binding was demonstrated by the greater percentage of liver T_3* bound to the nuclear fraction in the absence of carrier T_3 (4.75 versus 1.47) (Table 3). Saturable binding was shown to be mainly to intranuclear sites. This was demonstrated by the reduced proportion of radioactivity removed by sucrose buffer and Triton X-100 in the absence of carrier T_3. More specifically, in nuclei exposed to no carrier T_3, 23.1% of the radioactivity was lost by the sucrose buffer wash, 11.0% by the Triton X-100 wash leaving 65.9% in the residual pellet (Table 3). While for trout exposed to 10,000 ng carrier T_3 only 32.5% of the nuclear radioactivity remained in the residual pellet following buffer and Triton extraction.

Table 3.

The effects of extraction with sucrose buffer and Triton X-100 on the binding of T_3*to liver nuclei labelled in the presence of 0 or 10,000 ng T_3. Values represent the mean of 4 determinations. All values in the 0 ng T_3 group differ significantly from those in the 10,000 ng group ($P<0.01$).

fraction	0 ng T_3		10,000 ng T_3	
	% extracted	N / L	% extracted	N / L
Total	100.0	4.75	100.0	1.47
Sucrose buffer	23.1	1.10+	36.3	0.53+
Triton X-100	11.0	0.52	31.2	0.46
Residual pellet	65.9	3.13	32.5	0.48

+ N / L values estimated as (total N / L) x % in fraction.

Furthermore, T_3*-binding to nuclei in the absence of carrier T_3 was 3.2-fold higher than in the presence of high carrier T_3 when total binding was assayed and 6.5-fold higher after buffer and Triton extraction (Table 3).

II. Characterization of nuclear-bound T_3* in KCl extracts

An average of 5.11% of the liver radioactivity was in the nuclear fraction of trout receiving no carrier T_3 compared to 1.69% in trout receiving a carrier dose of T_3 (Table 4). In relation to the previous experiment using Triton X-100, the KCl extraction used here was more effective in removing nuclear-bound radioactivity from nuclei labelled in the absence of carrier T_3. KCl extracted 74.4% of the nuclear radioactivity from fish not exposed to carrier T_3. Of this extracted radioactivity 31.4% was bound to a macromolecule as it eluted in the void volume of G-25 Sephadex columns (Table 4). In contrast, fish receiving 10,000 ng carrier T_3 contained only 56.4% of the nuclear-bound T_3 in the KCl extract. Of this radioactivity extracted with KCl only 6.9% eluted in the macromolecular fraction. Both in the presence or absence of carrier T_3 less than 1.5% of the extracted radioactivity eluted in the iodide fraction. Furthermore, if macromolecular-bound T_3* is equated to the N/L ratio, only 0.07% of the liver T_3* elutes in the macromolecular fraction at high carrier T_3 doses compared to 1.18% in the absence of carrier T_3. It is concluded that a significant portion of the saturable nuclear binding is related to a macromolecule extracted with KCl buffer.

Elution of radioactivity in the macromolecular fraction was not an artifact resulting from the association of T_3* with macromolecules

Table 4.

The effects of extraction with KCl and filtration through G-25 Sephadex columns on T_3* bound to nuclei labelled in the presence of 0 or 10,000 ng T_3. MMB represents macromolecular-bound T_3*. Values represent the mean of 12 determinations. All values in the 0 ng T_3 group differ significantly from those in the 10,000 ng group ($P<0.01$).

fraction	0 ng T_3		10,000ng T_3	
	% extracted	N / L	% extracted	N / L
Total	100.0	5.11	100.0	1.69
Non-KCl extractable	25.6	1.31$^+$	43.6	0.74$^+$
KCl extractable	74.4	3.80	56.4	0.95
Non-MMB	51.0	2.61	52.5	0.88
MMB	23.4	1.20	3.9	0.07

$^+$ N / L value estimated as (total N / L) x % in fraction.

on the Sephadex column. In a separate experiment (Table 5), KCl extracts prepared from non-injected trout were passed through columns containing T_3* and variable doses of carrier T_3. In these cases 2.1% or less of the column T_3* eluted in the macromolecular fraction.

III. Stability of macromolecular T_3* complexes

Hormone binding was stable for 1 hour at $0^{o}C$, but the T_3*-macromolecular complex was destroyed within 1 hour by incubation at 25 or $37^{o}C$ (Table 6). The macromolecule to which T_3* was bound was identified as a protein (Table 7). Nucleases had negligible effects whereas proteolytic enzymes markedly reduced the amount of macromolecular-bound T_3*. A separate experiment verified that DNase and RNase were active under the conditions employed. By comparing the effectiveness of the three proteolytic enzymes one can determine the class of protein molecule responsible for saturable T_3-binding. Trypsin is relatively specific for the arginyl and lysyl residues characteristic of histone proteins (Lehninger, 1972; Surks et al., 1973). In contrast α-chymotrypsin and pronase have a wider spectra of action also exhibiting endopeptidase activity. A greater breakdown of macromolecular T_3* complexes with α-chymotrypsin and pronase, as compared to trypsin, suggest a high proportion of macromolecular-T_3* is bound to non-histone proteins.

D. Discussion

The present studies indicate that saturable T_3-binding sites consist of intranuclear proteins, probably non-histone in nature.

Differential labelling of liver nuclei evident when the carrier T_3 dose is varied, relates to the predominance of an intranuclear T_3*-binding site in the absence of carrier T_3. Losses of nuclear-bound T_3*

Table 5.

Macromolecular T_3* complexes formed by nuclear KCl extracts from uninjected trout. T_3* and T_3 were added to Sephadex columns in 0.3 ml of KCl buffer. Contaminating $^{125}I^-$ was removed by 5 ml of KCl buffer. A 0.2 ml sample of a common pool of extract was added to the column and incubated at $4^{o}C$ for 15 min. Macromolecular T_3* was removed by elution with 2 ml of KCl buffer, and was expressed as the percent of added T_3* corrected for iodide contamination. Values are the mean of closely-agreeing triplicate determinations.

	added T_3 (1×10^{-12} moles)			
	0	100	500	2000
% in macromolecular fraction	2.1	1.4	1.4	1.3

Table 6.

The effect of 1 hr incubation at various temperatures on the stability of macromolecular T_3* complexes. An unincubated group (0^oC) served as a control. Data are expressed as the mean of closely-agreeing triplicate determinations.

treatment	% T_3 in macromolecular fraction	% of control
control	31.5	----
0^oC	28.7	91.2
25^oC	15.0	47.6
37^oC	5.5	17.0

Table 7.

The effect of 1 hr incubation at $0^{o}C$ in the presence of various enzymes on the stability of macromolecular T_3* complexes. An incubated group containing no enzyme served as a control. Data are expressed as the mean of closely-agreeing triplicate determinations.

treatment	% T_3 in macromolecular fraction	% of control
control	28.7	----
DNase	25.5	88.8
RNase	31.0	107.9
trypsin	13.1	45.8
α-chymotrypsin	2.6	9.0
pronase	2.5	8.8

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following sucrose buffer extraction to remove weakly bound T_3* and Triton X-100 to remove the outer nuclear membrane were greatest at high carrier T_3 levels. This suggested saturable sites evident in the absence of carrier T_3 were intranuclear whereas non-saturable nuclear sites evident at high carrier T_3 doses exist on the nuclear membrane. While a single treatment with Triton X-100 may not remove the entire outer nuclear membrane, additional treatments were not feasible since extensive breakdown of nuclei occurs as monitored by DNA losses with more prolonged Triton X-100 exposure.

The trends found in this study were similar to those reported by Surks et al. (1973) using rat liver nuclei. Surks et al. (1973) also extracted nuclear globulins and ribonucleoproteins with negligible loss of bound radioactivity at low and high carrier T_3 levels, indicating that most of the saturable binding was associated with the crude chromatin fraction.

Macromolecule T_3* complexes extracted with 0.4M KCl were shown to be saturable by the reduced proportion of T_3* bound at high carrier T_3 levels. Similar macromolecule T_3 complexes have been reported by several authors using mammalian nuclear preparations. Mammalian macromolecule T_3 complexes bind to chromatin (reviewed in Chapter 2), however it is not known if a similar situation exists in fish. The effects of temperature and various enzymes on macromolecule T_3* complexes in the present study (Tables 6 and 7) were comparable to those reported for mammals (Surks et al., 1973; DeGroot et al., 1974; Samuels et al., 1974). Mammalian complexes were heat-labile, with extensive degradation occurring at 37°C. Surks et al. (1973) suggested

the saturable binding component was associated with the acidic protein fraction, based on enzyme treatments and a larger proportion of nuclear-bound T_3 extracted at pH 8.5 (76%) than at pH 6.0 (30%). Samuels *et al.* (1974) chromatographed KCl extracts of GH_1 cells labelled *in vitro* on Bio-Rex 70 columns which selectively elute non-histone and histone proteins. A radioactive peak was not evident in the histone fraction.

CHAPTER 6 - AFFINITY AND CAPACITY OF SATURABLE T_3- BINDING SITES

A. Objective

The previous experiments have indicated that saturable T_3-binding protein sites exist in trout liver nuclei. However, the binding properties of these sites are not known. The main objective in this Chapter has been to determine the affinity (equilibrium constant) for these saturable sites and their binding capacity.

A second objective has been to determine the effect of starvation on these properties of the nuclear sites. This was of relevance to the present study since all fish were starved for 3 days prior to use in experiments. Recent reports suggest that the capacity of saturable T_3-binding sites in rat liver nuclei is reduced during starvation (Burman et al., 1977; DeGroot et al., 1977a; Dillman et al., 1978b; Schussler and Orlando, 1978). A similar situation may exist in trout.

B. Protocol

I. Determination of nuclear-binding properties in 1-year-old trout

Idaho trout were maintained on a 1.7% food ration for 7 days and then starved 3 days prior to injection. Nine groups of 6-7 fish (n=62, mean wt. 40.9g, SEM 0.8) received intraperitoneal injections of both T_3* (0.30µCi, iodide contamination 3.7%) and a set dose of T_3 (0, 25, 50, 100, 150, 200, 250 or 1250 ng). Owing to the large number of fish involved, the injections were staggered over 2 days. Twelve hr after injection each fish was bled, killed and the nuclear fraction obtained. Technical problems prevented measurement of endogenous T_3 levels at the time this experiment was conducted. Plasma T_3 levels were obtained from 9 uninjected trout of comparable size held under similar conditions 13 days prior to this experiment.

II. Effects of starvation in 2-year-old trout

Idaho and British Columbia trout were randomly assigned to fed or starved treatments. Initially trout were maintained on a 1% food ration for 2-5 days. The group designated as fed received a 3.0% ration (1.5% ration twice a day) for 16 days. The group designated as starved was maintained on a 1.5% food ration for 13 days followed by 3 days of starvation. Subgroups of 7-8 trout received intraperitoneal injections of both T_3* (0.83μCi, iodide contamination 4.0%) and a set dose of T_3 (0, 25, 50, 125, 200, 400, 2,000, or 10,000 ng). Owing to the large numbers of fish involved, the injections were staggered over a 4 day period. Subgroups of 8-10 uninjected fish were bled each day to determine endogenous T_3 and T_4 levels. Twelve hr after injection each fish was bled, killed and the nuclear fraction obtained. Portions of each nuclei preparation were removed to determine DNA content.

C. Calculation of the Equilibrium Constant and the Capacity of Saturable Nuclear T_3-Binding Sites

The equilibrium constant and capacity of saturable nuclear T_3-binding sites were determined from a modified Wilkinson plot (see Appendix II for theory), in which the ratio of the free liver T_3 to the saturable nuclear-bound T_3 (ordinate) is plotted against the free liver T_3. From the resulting linear regression the equilibrium constant (k) is determined as the slope divided by the ordinate intercept and the binding capacity (M) is determined as the reciprocal of the slope.

Free liver T_3 and saturable nuclear-bound T_3 were determined as follows:

Free liver T_3 = Total liver T_3 - saturable nuclear-bound T_3 (moles/g liver)

Total liver T_3 = Plasma T_3 X L/P (moles/g liver)

where, $$\frac{L}{P} = \frac{T_3^* \text{ cpm/g liver}}{T_3^* \text{ cpm/g plasma}}$$

Plasma T_3 = Endogenous plasma T_3 + Exogenous plasma T_3 (moles/g plasma)

(Endogenous plasma T_3 was determined by RIA on plasma from uninjected trout maintained in a comparable manner to the injected fish; exogenous plasma T_3 was obtained from the product of the % injected dose of T_3^*/g plasma and the molar quantities of injected isotopic and carrier T_3).

Saturable nuclear-bound T_3 = Total liver T_3 (N/L obs. - N/L non-sat.) (moles/g liver)

where, $$\frac{N}{L} = \frac{T_3^* \text{ cpm in nuclear fraction/g liver}}{T_3^* \text{ cpm/g liver}}$$

N/L was determined for trout administered a particular set amount of carrier T_3 (N/L obs.) or for trout administered a high saturating level of carrier T_3 (N/L non-sat.). To verify that the high carrier levels of T_3 had indeed saturated the low-capacity sites, a Scatchard plot (see Appendix III for theory) was used. In this case, N/L (ordinate) is plotted against total nuclear-bound T_3 (Total liver T_3 X N/L). The resultant plot is biphasic. The initial linear phase

with negative slope corresponds to the saturable T_3-binding sites. The second phase, represented as a horizontal asymptote corresponds to the non-saturable T_3-binding. This horizontal asymptote provides the N/L non-sat. value.

Saturable nuclear-bound T_3 was routinely related to wet liver weight; in the second experiment it was also related to the DNA content of the liver sample.

The following assumptions have been made in these calculations:

(i) T_3 does not deiodinate and is not otherwise converted or degraded.
(ii) Over the period of measurement a steady state exists with respect to T_3 metabolism.
(iii) Isotopic equilibrium prevails at the time T_3* determinations are made.
(iv) Free hormone includes all liver T_3 that is not bound to saturable nuclear sites.
(v) Diffusion kinetics apply and no active transport of T_3 takes place.

D. Results

I. Determination of nuclear-binding properties in 1-year-old trout

Due to the logistics of handling the large number of fish, injections were staggered over 2 days. To assess the effects of staggered injection two groups of trout injected with both T_3* and 0 ng T_3 28 hr apart were compared. The staggered injection protocol did not alter the distribution of T_3* in these groups (Table 8), and for subsequent determination of the capacity and affinity they were combined.

With progressive increases in carrier T_3 levels the N/L values declined as shown in the Scatchard plot (Fig. 4A). Saturation of low-capacity sites occurred at a total nuclear T_3 concentration of 2.7×10^{-12} mole/g liver and corresponded to a N/L ratio of 0.021.

Table 8.

Comparison of body weight and tissue uptake of T_3* in trout injected with T_3* and 0 ng T_3 28 hr apart and killed 12 hr after injection. (n: 0 hr = 7; 28 hr = 6). No significant differences were evident using a paired t-test ($P>0.05$).

	0 hr		28 hr	
	$\bar{x}$	SD	$\bar{x}$	SD
Weight (g)	39.53	6.74	40.60	6.59
Plasma (% dose/g)	7.18	1.89	7.11	1.55
Liver (%dose/g)	15.98	3.89	19.87	6.20
L / P	2.28	0.55	2.78	0.53
Nuclei (% dose/g)	0.61	0.15	0.79	0.33
N / L	0.0369	0.0047	0.0392	0.0059
Gall bladder (% dose)	9.15^{+}	2.83	13.48	4.82

$^{+}$ n = 6.

Figure 4. T_3-binding to saturable sites in liver nuclei of 1-year-old trout.

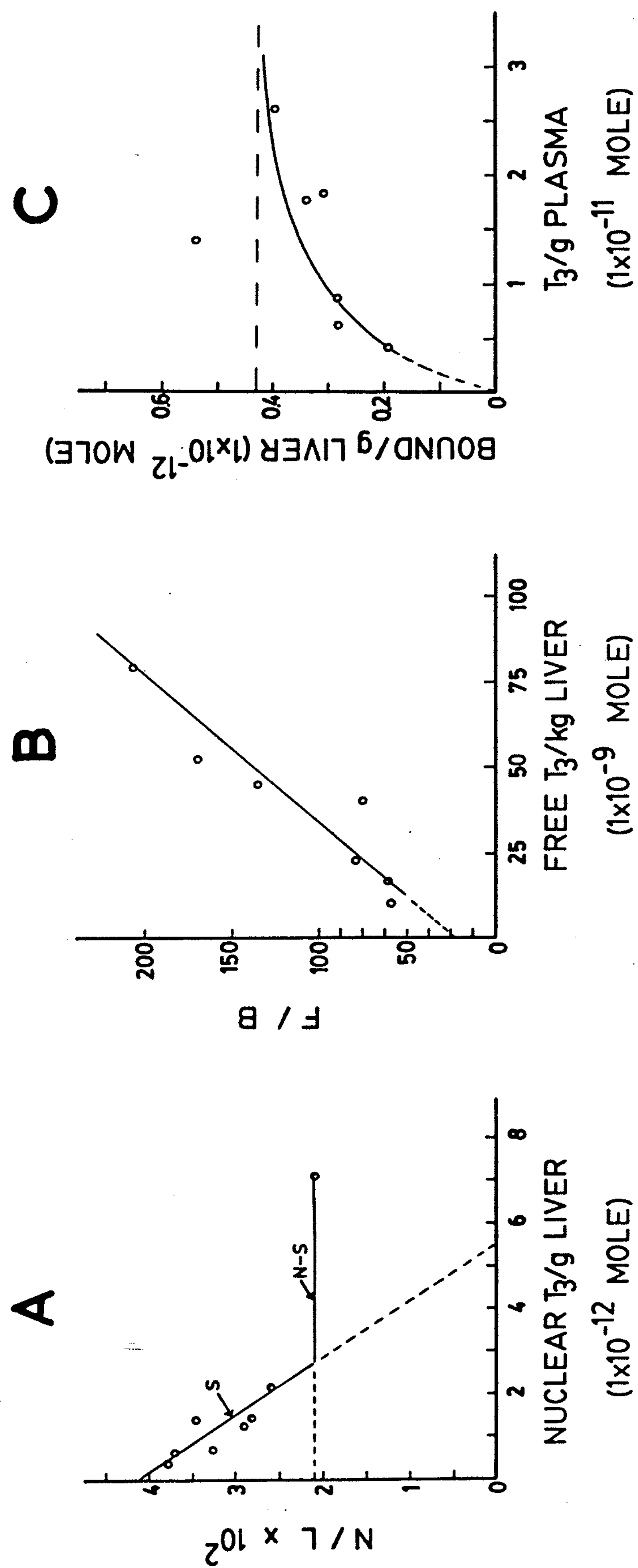
A. Scatchard plot demonstrating saturable (S) and non-saturable (N-S) binding sites.

B. Wilkinson plot. The regression equation was:

$$y = (2.30 \times 10^8 \text{ kg liver/mole } T_3)\ x + 22.9.$$

C. Saturable T_3-binding/g liver as a function of plasma T_3 level. The binding capacity was included as a broken line.

(For original data see Appendix IV.)


A
N / L x 10^2
S
N-S
NUCLEAR T_3/g LIVER
($1x10^{-12}$ MOLE)
B
F / B
FREE T_3/kg LIVER
($1x10^{-9}$ MOLE)
C
BOUND/g LIVER ($1x10^{-12}$ MOLE)
T_3/g PLASMA
($1x10^{-11}$ MOLE)

From these values and the average L/P ratio, the level of plasma T_3 necessary to saturate nuclear sites was determined. In this case the average L/P ratio was 2.76 and the corresponding saturating plasma concentration was 46.7×10^{-12} mole/g plasma. This value was about 11-fold higher than the estimated endogenous T_3 level of 4.2×10^{-12} mole/g plasma.

Following correction for non-saturable binding, the properties of the saturable sites were determined from a Wilkinson plot (Fig. 4B). A binding capacity of 0.43×10^{-9} mole T_3/kg liver and an equilibrium constant of 1.0×10^{8} kg liver/mole T_3 were derived.

The amount of T_3 bound to saturable sites was related to the estimated plasma T_3 concentration (Fig. 4C). As the plasma T_3 concentration increased, binding to saturable sites increased reaching a plateau, corresponding to the capacity. It was estimated from Fig. 4C that 45% of the saturable sites were occupied at endogenous T_3 levels. In this calculation, the amount bound in the absence of carrier T_3 was considered to represent that at endogenous T_3 levels as plasma T_3 levels were negligibly influenced (<2%) by just T_3* injection.

II. Effects of starvation in 2-year-old trout

A lower nutritional level followed by starvation for 3-days did not influence body weight or DNA content of the entire liver, but significantly depressed liver weight, hepatosomatic index (HSI), and plasma T_4 and T_3, while elevating the DNA content per gram liver (Table 9). No effect due to gender or state of sexual maturity was apparent.

Uptake of T_3* into the nuclear fraction was dose-dependant in both fed and starved groups. This dose dependance was reflected in

Table 9.

Influence of feeding or starvation on body weight and several liver and plasma parameters. Values for both T_3* injected and uninjected trout are shown. Statistical comparisons were made using a two-tailed t-test (not significant, when $P>0.05$).

	fed			starved		
	n	$\bar{x}$	SD	n	$\bar{x}$	SD
INJECTED						
Body weight (g)	64	141.84	29.18	63	137.61	23.98
Liver weight (g)	64	2.00	0.86	63	1.41^{+}	0.59
HSI	64	1.38	0.43	63	1.00^{+}	0.27
DNA nuclei fraction (mg/g liver)	64	1.67	0.77	63	2.23^{+}	0.64
DNA nuclei fraction (total liver, mg)	64	3.05	1.27	63	3.02	0.14
UNINJECTED						
Body weight (g)	20	145.84	31.46	17	138.09	19.07
Plasma T_4 (ng/ml)	19	3.52	1.30	17	1.89^{+}	1.37
Plasma T_3 (ng/ml)	20	2.88	1.27	17	1.96	0.73

$^{+}$ Significantly different from fed group, $P<0.01$.

distinct saturable and non-saturable components by Scatchard analysis (Fig. 5A). Saturating plasma T_3 levels were 27.2 X 10^{-12} mole T_3/g plasma in fed trout and 22.5 X 10^{-12} mole T_3/g plasma in starved trout (Table 10). These values represent a 6-fold increase above endogenous plasma T_3 levels in fed trout and a 7.5-fold increase above endogenous plasma T_3 levels in starved trout.

Wilkinson plots indicated that saturable liver nuclei sites from both fed and starved fish had similar equilibrium constants, capacities and occupancies at endogenous T_3 levels (Fig. 5B and Table 11). Similar equilibrium constants suggest that the properties of the binding site are not altered by a change in the daily food ration.

While T_3-binding capacity can in most instances be reliably related to liver weight, a problem arises during periods of altered food intake when the liver may change in size due to the mobilization of glycogen or other reserves without involving a proportional change in cell or nuclei number. Under these conditions the T_3-binding capacity is better related to DNA content, which will more adequately reflect the nuclear component. Saturable binding in fed and starved fish in relation to liver weight (Fig. 6A) or DNA content (Fig. 6B) has been plotted as a function of plasma T_3 concentration. The capacities of the saturable sites are also shown. The capacity in relation to DNA content, was calculated from the product of the capacity determined from the Wilkinson plot and the average DNA content of the nuclear pellet. The capacity in fed trout was 0.37 X 10^{-12} mole T_3/mg DNA compared to 0.28 X 10^{-12} mole T_3/mg DNA in starved trout. In relation to liver weight, fed and starved trout had

Figure 5. T_3-binding to saturable sites in liver nuclei of fed and starved 2-year-old trout.

A. Scatchard plots demonstrating saturable (S) and non-saturable (N-S) binding sites.

B. Wilkinson plots. The regression equation for fed trout was:

$$y = (1.64 \times 10^8 \text{ kg liver/mole } T_3)\ x + 17.8.$$

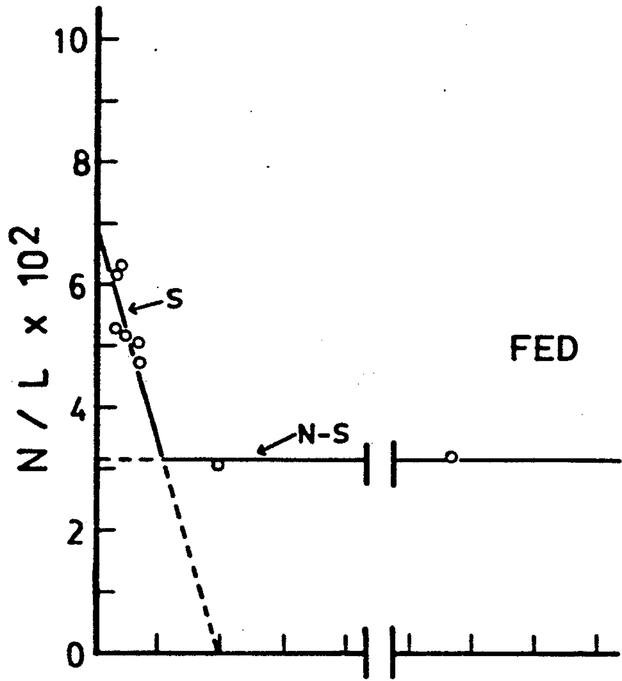
The regression equation for starved trout was:

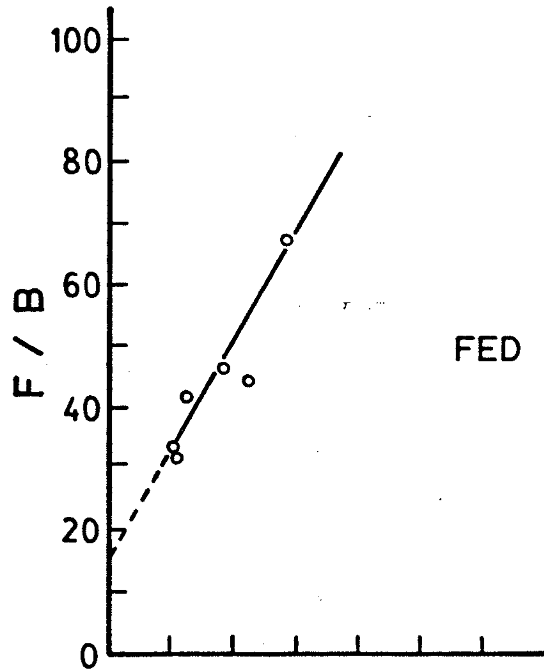
$$y = (1.62 \times 10^8 \text{ kg liver/mole } T_3)\ x + 13.6.$$

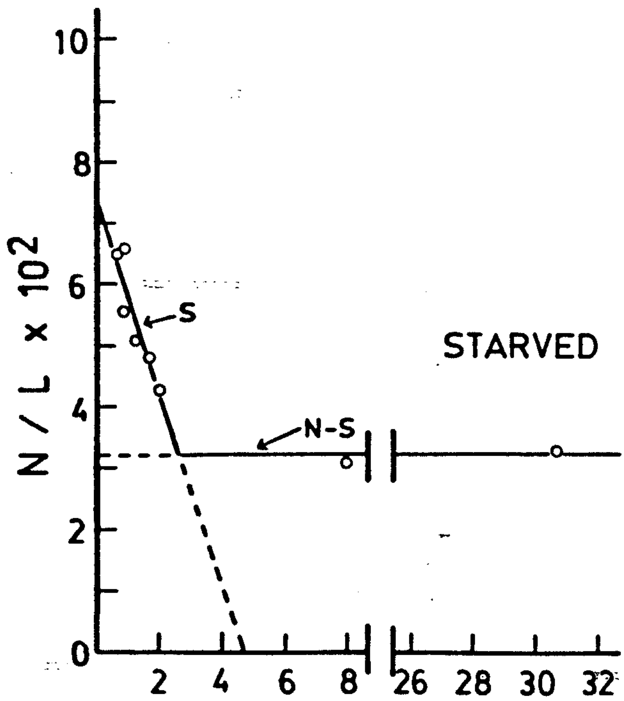
(For original data see Appendix V.)

A

B







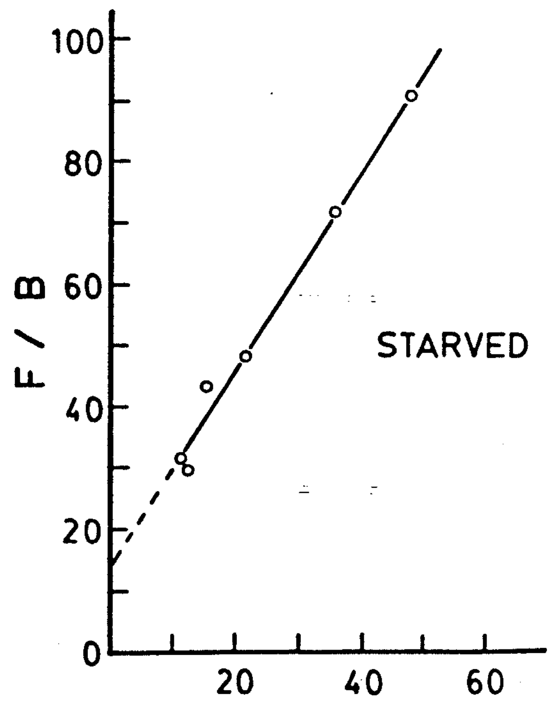


Table 10.

Parameters used in the estimation of plasma T_3 levels required to saturate the high-affinity low-capacity T_3-binding sites in fed and starved trout.

parameter	fed	starved
Nuclear T_3 sat. (mole/g)$^{+}$	2.0×10^{-12}	2.6×10^{-12}
N / L non-sat.$^{+}$	0.0314	0.0320
Liver T_3 sat. (mole/g)$^{++}$	63.7×10^{-12}	81.3×10^{-12}
L / P	2.34	3.61
Plasma T_3 sat. (mole/g)$^{++}$	27.2×10^{-12}	22.5×10^{-12}
Endogenous plasma T_3 (mole/g)	4.5×10^{-12}	3.0×10^{-12}

$^{+}$ From Scatchard plot (Fig. 5A).

$^{++}$ Where,

$$\text{Liver } T_3 \text{ sat.} = \frac{\text{Nuclear } T_3 \text{ sat.}}{\text{N / L non-sat.}}$$

and

$$\text{Plasma } T_3\text{-sat.} = \frac{\text{Liver } T_3 \text{ sat.}}{\text{L / P}}$$

Table 11.

Calculated equilibrium constant (k), binding capacity (m) and proportion of saturable sites occupied at endogenous T_3 levels for starved and fed trout.

condition	k (1×10^{8} kg liver/mole T_3)	m (1×10^{-9} mole T_3/kg liver)	occupancy (%)
Starved	1.2	0.62	58
Fed	0.9	0.61	50

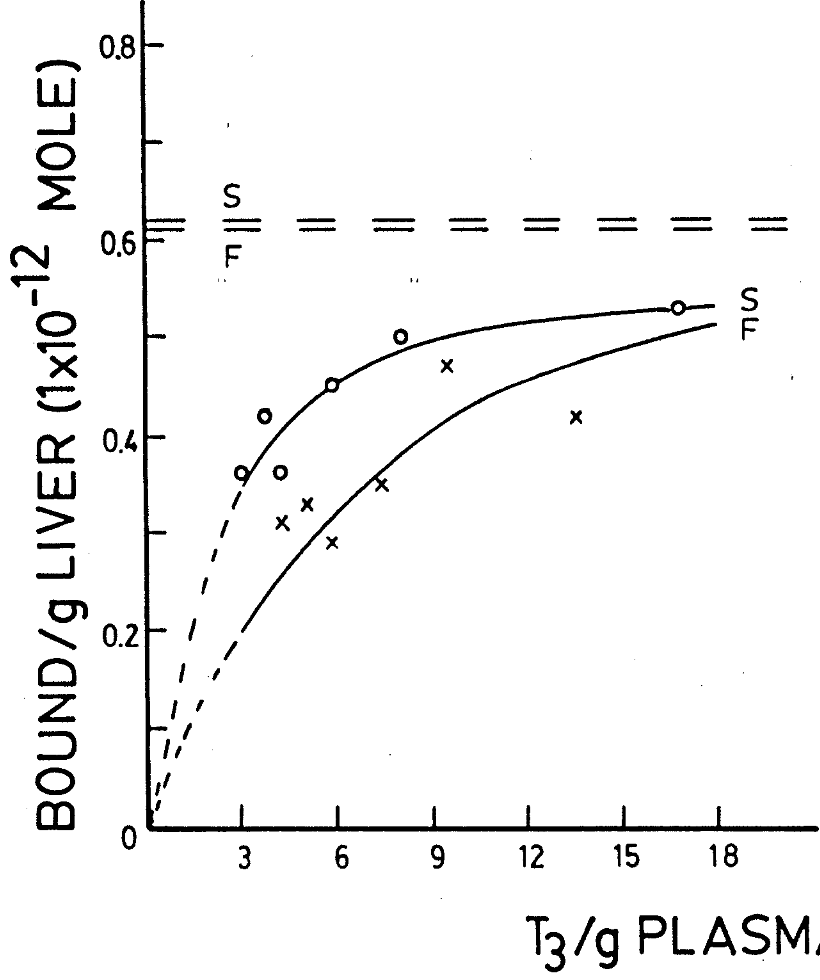
Figure 6. T_3-binding to saturable sites in liver nuclei as a function of plasma T_3 levels in fed (F) and starved (S) 2-year-old trout.

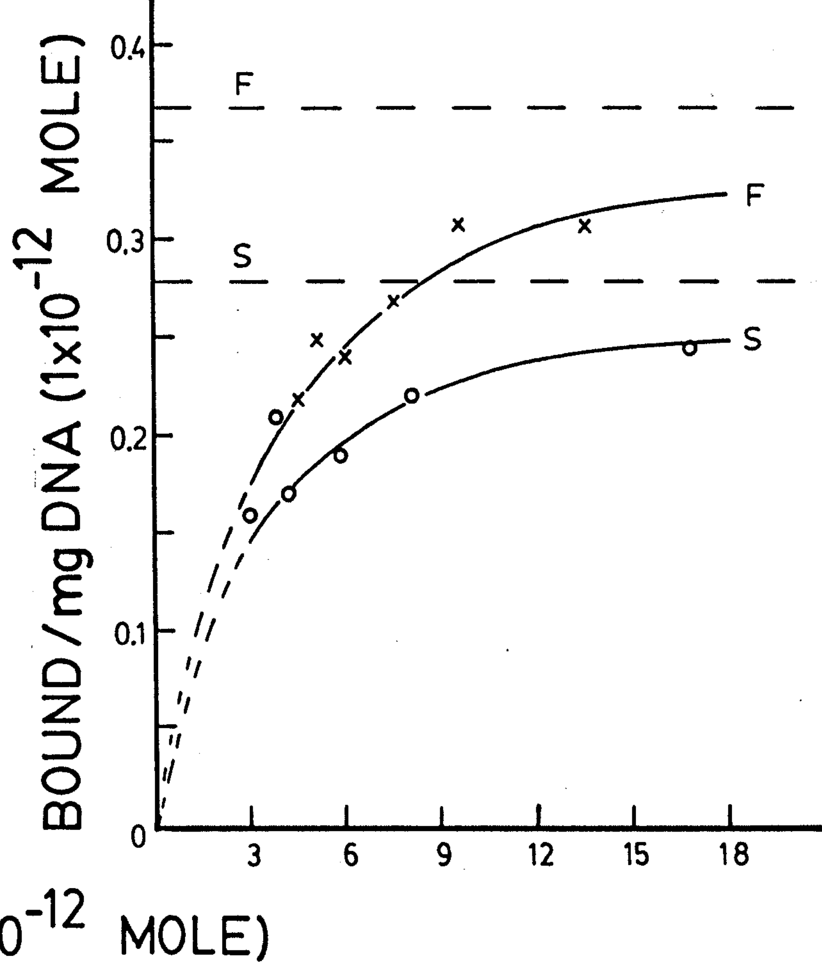
A. Saturable binding/g liver.

B. Saturable binding/mg DNA.

Binding capacities are indicated by the broken lines.

(For original data see Appendix V.)


A
BOUND/g LIVER (1×10^{-12} MOLE)
0
0.2
0.4
0.6
0.8
S
F
3
6
9
12
15
18
T_3/g PLASMA (1×10^{-12} MOLE)


B
BOUND/mg DNA (1×10^{-12} MOLE)
0
0.1
0.2
0.3
0.4
F
S
3
6
9
12
15
18

similar T_3-binding capacities, while in relation to DNA content the binding capacity was reduced by 24% in starved trout.

While starvation reduces the capacity, this reduction will only be of physiological consequence if the number of binding sites occupied by T_3 is reduced at endogenous plasma T_3 levels. Figure 6B indicates that fed fish with an endogenous T_3 level of 4.5×10^{-12} mole T_3/g plasma had 0.22×10^{-12} mole T_3 bound to saturable sites/mg DNA, while starved trout with an endogenous T_3 level of 3.0×10^{-12} mole T_3/g plasma had 0.16×10^{-12} mole T_3 bound/mg DNA. Thus starvation has reduced the number of saturable T_3-binding sites occupied at endogenous T_3 levels by 37%.

E. Discussion

Saturable T_3-binding sites in trout liver nuclei are characterized by low capacity but high affinity. The properties of the saturable sites in the present study are comparable to those of saturable sites in rat liver nuclei in which binding has been correlated with metabolic effects.

The equilibrium constant of saturable binding sites in trout averaged 1.0×10^{8} kg liver/mole T_3, a value similar to the 1.4×10^{8} kg rat liver/mole T_3 calculated in the same manner (Oppenheimer _et al._, 1974a). It should be stressed, that the equilibrium constants in the present study and for the rat are not association constants, since the equilibrium was assessed between the nuclei and cytoplasm, and not between the nuclei and an artificial aqueous medium (Oppenheimer _et al._, 1974a). Similar equilibrium constants in the trout and rat reflect a similar partition between extranuclear and saturable nuclear sites, and

may be a consequence of binding to comparable proteins (Chapter 5). However, the magnitude of the association constant governing these sites may differ in the trout and rat. Firstly, the kinetic properties in trout were based on measurements made at 12°C whereas for rats measurements were made at 37°C. Secondly, the T_3-binding affinity of extranuclear sites may differ, based on the different pattern of T_3* uptake into the liver of trout and rats (Chapter 4).

The capacity of saturable sites for trout, in relation to liver weight or DNA content, was about 3-fold lower than comparable values for rat liver. Capacities in fed rats were 1.8×10^{-9} mole T_3/kg liver or 1.1×10^{-12} mole T_3/mg DNA (Oppenheimer et al., 1974a). It is difficult to assess the significance of the lower capacity in trout. In the rat capacity varies between T_3-responsive and non-responsive tissues (Oppenheimer et al., 1974b). While trout liver is prominent in accumulating T_3 (Chapter 4), it is not known how it fits in the hierarchy of T_3-responsive tissues, or if the role of T_3 in trout liver is the same as for the case of rat liver.

In the present study, and those on the rat, binding capacities were not corrected for the loss of nuclear material incurred during the isolation procedure. Assuming a constant 41% loss of DNA and correcting for these losses the binding capacities range from 0.73 - 1.05×10^{-9} mole T_3/kg liver in 1-and 2-year-old trout. In the rat, recovery of DNA was 62% (Oppenheimer et al., 1974a).

In trout capacity, in contrast to affinity, is dependant on age and/or body weight. Capacities in starved and fed 2-year-old trout were about 140% higher than for trout one year younger and 100g less in

body weight. An age-dependant increase in the binding capacity was observed for saturable binding sites in rat liver nuclei measured *in vitro*, where the binding capacity quadruples from birth to 50 days (DeGroot *et al*., 1977b). Binding capacities measured in relation to DNA content were reduced by 24% in starved trout. For the rat, starvation for 1-5 days reduced the binding capacity measured *in vitro* by 30-50% without change in the association constant (Burman *et al*., 1977; DeGroot et al., 1977a; Dillman *et*. *al*., 1978b; Schussler and Orlando, 1978). Factors contributing to a change in the number of saturable binding sites are not known. This change may reflect differential rates of synthesis or degradation of saturable binding sites or a combination of these events.

At endogenous T_3 levels in trout about 50% of the saturable sites contain T_3. Complete occupancy of saturable sites requires a 6-11-fold increase of plasma T_3 above endogenous levels. However, the amounts of T_3 bound to saturable sites could be modulated by modest changes in the plasma T_3 concentration (Fig. 4 and 6).

At present no data are available to link binding to saturable sites in trout and subsequent metabolic events. The association of T_3 with non-histone proteins in trout may be significant as this class of protein has been considered to regulate specific gene function. However, the reduced binding to saturable sites at endogenous T_3 levels during starvation provides a focal point for speculation on the role of these sites in trout. Some actions of thyroid hormones in trout have been associated with metabolically costly events such as increased protein synthesis (Narayansingh and Eales, 1975) and growth

(reviewed by Eales, 1978). Of significance to the present study were findings that reduced food intake resulted in decreased conversion of T_4 to T_3 in brook trout (Higgs and Eales, 1977 and 1978). Assuming that in fish T_3 is the more metabolically potent thyroid hormone, as appears to be the case in mammals, this reduced production of T_3 in the peripheral tissues may provide a mechanism to reduce the metabolic consequences of T_3 when caloric reserves are low. In the present study reduced food intake and starvation for 3 days resulted in a significant depression of both T_4 and T_3 levels in the plasma and a 37% reduction in the amount of T_3 bound to saturable sites at endogenous T_3 levels. Reduced binding to saturable sites may provide the cellular basis for the control of tissue effects when caloric reserves are low. If T_3-binding to saturable sites is involved with the stimulation of nuclear events, simultaneous determination of the amount bound to saturable sites and nuclear response will allow a more constructive evaluation of their significance.

It is necessary to comment on the validity of the kinetic assumptions made in this study. (i) Liver radioactivity was assumed to represent T_3. Deiodination of injected T_3* was negligible (Chapter 4). Chromatography of liver ethanol extracts gave no indication of T_3 conversion to other products (Eales and Van Der Kraak, unpublished). (ii) Steady state conditions with respect to T_3 metabolism were assumed. Brown _et al_. (1978) found that limited stress due to anesthetization and injection did not influence plasma T_3 levels. Furthermore, liver uptake measured 12 hr after injection was not affected by the carrier T_3 dose (Table 2 and 12). (iii) Isotopic

Table 12.

One-way ANOVA for liver T_3* uptake at varied carrier T_3 levels in 1- and 2-year-old trout. (Not significant, NS, when $P>0.05$).

group	n	Bartlett's X^2	significance	ANOVA F-test	significance
1-YEAR-OLD					
Starved	62	5.49	NS	0.69	NS
2- YEAR-OLD					
Fed	64	12.61	NS	0.52	NS
Starved	63	4.23	NS	1.28	NS

equilibrium was assumed. Sampling at 12 hr represented the midpoint of the nuclear uptake plateau, and at this time the liver and plasma were acting as a single kinetic compartment (Chapter 4). (iv) It was assumed all liver T_3 was available for binding to saturable nuclear sites. While this may be the case in classical models of hormone-receptor studies, such as the binding of hormones by plasma proteins in vitro, it need not be the case in vivo. However no evidence for high-affinity sites in other subcellular fractions was found (Chapter 4). (v) Diffusion kinetics were assumed to govern the interaction of T_3 with saturable nuclear sites. To date no evidence has been reported to suggest protein-mediated or energy-dependant transfer of T_3 to nuclear sites in the rat. However, Krenning et al.(1978) demonstrated the active transport of T_3 into isolated rat liver cells. It is not known if similar situations exist in fish. If energy-dependant liver uptake occurs in fish, the transport system was not saturated at the high carrier T_3 doses used in this study.

Lastly, the demonstration of saturable T_3-binding sites poses more questions than it answers. Are T_3-nuclear interactions specific? For example, does T_4, present in comparable levels in plasma, also bind to nuclear sites? Attempts to characterize T_4-binding to nuclei are hampered by extensive deiodination of T_4 to T_3 in vivo and the lack of a suitable in vitro nuclear binding system. While T_4 competes to a limited degree with T_3-binding sites in mammals it is hard to estimate the effect of T_4 in fish.

Binding to saturable nuclear sites constitutes only a small percentage of the liver T_3. Were saturable sites overlooked in other

subcellular fractions by the presence of non-saturable binding? Do hormonal effects necessitate binding to high-affinity sites or are the actions of T_3 a multiple of effects involving low-affinity and high-affinity sites? Additional research is necessary to clarify these questions.

CHAPTER 7 - SUMMARY AND CONCLUSIONS

1. The liver concentrated T_3* to a greater extent than other trout tissues and was selected as the tissue best suited for studying nuclear binding.

2. Maximal uptake of T_3* into the liver nuclear fraction was evident 8-16 hr after intraperitoneal T_3* injection. Subsequent studies for characterization of nuclear T_3-binding sites were conducted 12 hr after T_3* injection when isotopic equilibrium was assumed.

3. Saturable T_3-binding sites were demonstrated in the nuclear fraction by the reduced binding of T_3* in the presence of high levels of carrier T_3. Only non-saturable binding sites were evident in the mitochondrial, microsomal or cytosol fractions as no displacement of T_3* was found with a high carrier T_3 dose.

4. Saturable T_3-binding relates to an intranuclear binding site as removal of the outer membrane had little effect on bound T_3*. Non-saturable nuclear binding occurred predominantly on the outer nuclear membrane.

5. A significant proportion of the nuclear-bound T_3* extracted with 0.4M KCl behaved as a macromolecule when filtered through G-25 Sephadex columns. High levels of carrier T_3 reduced the proportion of macromolecular-bound T_3*, indicating the saturability of these complexes. The macromolecule was identified as a heat-labile protein, probably non-histone in nature.

6. Saturable T_3-binding sites were characterized by high affinity and low capacity. The equilibrium constant was comparable for 1-and 2-year-old trout ranging from $0.9 - 1.2 \times 10^8$ kg liver/mole T_3.

Binding capacity ranged from 0.43 X 10^{-9} mole T_3/kg liver for starved 1-year-old trout to 0.62 X 10^{-9} mole T_3/kg liver for starved 2-year-old trout.

7. From 45 to 58% of the available T_3-binding sites were occupied at endogenous T_3 levels. Binding sites were saturated when endogenous T_3 levels were increased 6-11-fold.

8. In relation to DNA content, binding capacity in starved 2-year-old trout was reduced by 24% when compared to fed trout. This was accompanied by a 37% reduction of the amount bound in starved trout at endogenous T_3 levels.

9. The significance of saturable T_3-binding sites in trout liver is not known. However, the properties of these sites closely parallel the properties of saturable T_3-binding sites in mammals, which are involved in the regulation of metabloic response. Reduced binding to saturable sites when caloric reserves are low may provide a cellular control mechanism to reduce the anabolic effects of T_3.

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APPENDICES

Appendix I.

T_3* and ^{125}I elution patterns from KCl-primed Sephadex columns. The percent of added T_3* and ^{125}I in sequential eluates with KCl buffer and human plasma in KCl buffer from Sephadex columns equilibrated to pH 7.6 with KCl buffer. Values represent the mean of 6 closely-agreeing determinations.

	KCl buffer (ml)			human plasma in KCl buffer (ml)			Recovery(%)
	2	3	2	4	4	4	
T_3*	0.2	3.8+	0.2	89.2	4.7	0.4	98.5
^{125}Iodide	10.8	81.6	1.5	1.9	0.1	0.1	96.0

+ Iodide contamination determined using barbital buffer = 4.3%.

Appendix II.

Wilkinson plot to determine affinity and capacity of saturable sites.

Oppenheimer et al. (1974a) proposed a model for describing T_3 interactions with saturable rat liver nuclear sites labelled in vivo. Their model is based on the following relationship:

$$T_3R + N \rightleftharpoons T_3N \qquad (1)$$

where T_3R is the concentration of T_3 in the cytoplasm, T_3N is the amount of T_3 bound to saturable sites, and N is the concentration of unoccupied saturable sites. This reaction is described by an equilibrium constant k, where

$$k = \frac{T_3N}{(N)\ (T_3R)} \qquad (2)$$

Now, by combining the equation of the expression of k, with the expressions for the conservation of mass,

$$\text{Total liver } T_3 = T_3R + T_3N \qquad (3a)$$

$$\text{Total Receptor} = M = N + T_3N \qquad (3b)$$

and substituting in equation (2) with rearrangement of terms the following equation is obtained,

$$\frac{T_3R}{T_3N} = T_3R\ \frac{1}{M} + \frac{1}{kM} \qquad (4)$$

From the slope and ordinate intercept of a plot of T_3R/T_3N as a function of T_3R, the binding capacity M (1/slope) and the equilibrium constant k (slope/ordinate intercept) can be determined.

Equation (4) is analogous to the Eadie or Wilkinson inversion plot of enzymology (Rodbard, 1973).

Appendix III.

Scatchard plot to distinguish saturable and non-saturable binding.

Scatchard analysis provides a graphical method to distinguish ligand binding to saturable and non-saturable binding sites (Balieu and Raynaud, 1970; Rodbard, 1973). Saturable sites are distinguished by reduced labelled ligand binding as carrier ligand increases whereas non-saturable sites bind a constant amount of labelled ligand regardless of the carrier ligand dose. Therefore, in the present study by relating the proportion bound, N/L, to the quantity of T_3 bound (total nuclear bound T_3) a distinction between saturable and non-saturable sites can be made. The constant proportion of T_3* bound at high carrier T_3 doses corresponds to non-saturable binding represented by a horizontal asymptote on a Scatchard Plot. The decreasing proportion of T_3* bound at low carrier T_3 doses corresponds to saturable binding represented by a phase with negative slope.

Furthermore, the intercept of the non-saturable and saturable components represents the lower limit of displaceable T_3*. At this concentration saturable sites will contain only carrier T_3. By extrapolating to the absicca the concentration of nuclear T_3, and ultimately plasma T3 required to saturate nuclear sites, can be determined.

Appendix IV.

Saturable T_3-binding to liver nuclei in starved 1-year-old trout. Values represent the mean of 7 fish per dose (13 in the 0 ng group).

Injected dose (ng)	Plasma T_3/g (1×10^{-12} mole)	Liver T_3/g (1×10^{-12} mole)	N / L (%)	Corrected N / L (%)	Sat. nuclear T_3 /g liver (1×10^{-12} mole)
0	4.2	11.3	3.79	1.70	0.19
25	6.2	17.0	3.72	1.63	0.28
50	8.6	23.2	3.25	1.16	0.28
100	14.4	40.7	3.44	1.34	0.53
150	17.7	45.0	2.88	0.79	0.33
200	18.4	52.8	2.80	0.71	0.31
250	26.3	83.3	2.59	0.50	0.40
1250	137.5	352.2	2.09	----	----

Appendix V.

Saturable T_3-binding to liver nuclei in fed and starved 2-year-old trout. Values represent the mean of 8 fish per dose (7 in the starved 10,000 ng group).

	Injected dose (ng)	Plasma T_3/g (1×10^{-12} mole)	Liver T_3/g (1×10^{-12} mole)	N / L (%)	Corrected N / L (%)	Sat. nuclear T_3 /g liver (1×10^{-12} mole)	Sat. nuclear T_3 /mg DNA (1×10^{-12} mole)
FED	0	4.5	10.8	6.19	3.04	0.31	0.22
	25	5.1	10.9	6.26	3.11	0.33	0.25
	50	6.0	12.4	5.24	2.69	0.29	0.24
	125	7.5	18.5	5.12	1.97	0.35	0.27
	200	9.5	23.7	5.08	1.93	0.47	0.31
	400	13.6	28.7	4.74	1.59	0.42	0.31
	2,000	64.2	134.3	3.08	----	----	----
	10,000	383.2	910.9	3.20	----	----	----
STARVED	0	3.0	11.9	6.52	3.32	0.36	0.16
	25	3.8	12.9	6.57	3.36	0.42	0.21
	50	4.2	16.1	5.53	2.33	0.36	0.17
	125	5.9	22.2	5.06	1.86	0.45	0.19
	200	8.1	36.5	4.85	1.66	0.50	0.22
	400	16.8	48.3	4.26	1.06	0.53	0.24
	2,000	81.4	258.7	3.10	----	----	----
	10,000	448.1	1066.5	3.31	----	----	----