THE UNIVERSITY OF MANITOBA

INFLUENCE OF AMBIENT 3,5,3'-TRIIODO-L-THYRONINE

(T_3) ON PLASMA T_3, HEPATIC NUCLEAR T_3 BINDING

AND CERTAIN PHYSIOLOGIC RESPONSES IN RAINBOW

TROUT, SALMO GAIRDNERI, RICHARDSON

by

ROBERT JOHN OMELJANIUK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

WINNIPEG, MANITOBA

March, 1983

INFLUENCE OF AMBIENT 3,5,3'-TRIIODO-L-THYRONINE

(T_3) ON PLASMA T_3, HEPATIC NUCLEAR T_3 BINDING

AND CERTAIN PHYSIOLOGIC RESPONSES IN RAINBOW

TROUT, SALMO GAIRDNERI, RICHARDSON

BY

ROBERT JOHN OMELJANIUK

A thesis 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

© 1983

ABSTRACT

Immersion of rainbow trout (<u>Salmo</u> <u>gairdneri</u>) in the thyroid hormone, T_3 (3,5,3'-triiodo-L-thyronine), was evaluated as a means of chronically elevating plasma T_3 levels, altering occupancy of potential T_3 receptor sites and thereby eliciting changes in physiological events at the cellular level.

Immersion of trout in various concentrations of T_3 at 9-12°C, predictably elevated plasma T_3 levels, providing a method for the chronic elevation of plasma T_3. Most adjustments in plasma T_3 occurred by 48 hr with a steady state achieved by 5 to 7 days. Immersion in $[^{125}I]T_3$ (*T_3) showed that fractional plasma T_3 loss by biliary excretion was similar over a range of ambient T_3 concentrations.

An <u>in</u> <u>vivo</u> isotope displacement technique, involving immersion of trout in carrier T_3 followed by intraperitoneal injection of *T_3, was developed to describe saturable liver nuclear T_3-binding sites and their occupancy by T_3 under conditions of chronic T_3 immersion. Over 96% of nuclear radioactivity was attributed to *T_3. Maximal saturable binding of *T_3 to liver nuclear sites occurred 12 hr after *T_3 injection. The equilibrium constant (K) was estimated as 5.88×10^7 kg liver/mole T_3 (1.71×10^8 gDNA/mole T_3), and maximum binding capacity (MBC) as

i

389 pmole T_3/kg liver (134 pmole T_3/g DNA). The
relationships between ambient T_3, plasma T_3 and nuclear
T_3 occupancy were determined. At artificially-induced
plasma T_3 levels from 0.2-5.2 pmole/ml, nuclear occupancy
ranged from 4-55%; 100% nuclear saturation occurred at plasma
T_3 levels between 5 and 43 pmole/ml when ambient levels
were 2-10 μg T_3/100 ml (30-150 pmole/ml).

Chronically elevated plasma T_3 caused a significant
increase in liver Na^+/K^+-ATPase activity, a significant
decrease in gill ATPase and no significant change in
integumentary guanine content.

In conclusion, trout immersed in T_3 had predictably
increased plasma T_3 and occupancy of saturable liver
nuclear T_3-binding sites. Immersion increased liver
Na^+/K^+-ATPase and decreased gill Na^+/K^+-ATPase activity
but did not significantly influence integumentary guanine
content

TO MY MOTHER

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

Table of Contents (Cont'd)

Table of Contents (Cont'd)

LIST OF TABLES

LIST OF FIGURES

List of Figures (Cont'd)

FIGURE   PAGE

LIST OF APPENDICES

INTRODUCTION

Thyroid hormones have been postulated to elicit
their effects by interacting with a nuclear non-histone
protein binding site (receptor) (Seelig et al. 1981a).
Saturable nuclear T_3-binding sites have been found in
tissues of mammals (Oppenheimer et al., 1972), birds
(Bellabarba and Lehoux, 1981), amphibians (Galton, 1980),
and fish (Van der Kraak and Eales, 1980; Darling et al.,
1982). In mammals, saturable nuclear T_3-binding sites
have been demonstrated in a variety of rat tissues including:
liver, kidney, pituitary, heart, brain, spleen, and testis
(Oppenheimer et al., 1974a, b; DeGroot and Stausser, 1974)
and in pituitary tumour (GH-1) cell cultures (Samuels and
Tsai, 1973).

The interaction of T_3 with nuclear receptors stimulates
specific protein synthesis in the target tissues. This has
been demonstrated in GH-1 cells 45-60 min after T_3 binding
to receptors (Samuels and Shapiro, 1976). T_3 also stimulates
growth hormone production in cultured pituitary (GC) cells
(Martial et al., 1977b) and malic enzyme and α-glycerophosphate
dehydrogenase synthesis in rat liver (Oppenheimer et al., 1977).
In mammals, thyroid hormones are implicated in inducing
Na^+/K^+-ATPase (Smith and Edelman, 1979; Haber and Loeb,
1982; Garg et al., 1982). However, there is some evidence
that thyroid hormones do not exert the same effects on
toad (Bufo marinus) bladder Na^+/K^+-ATPase (Geering et al.,
1982).

1

Few data are available on saturable thyroid hormone binding sites in fish. Using an in vivo approach Van der Kraak and Eales (1980) described saturable liver nuclear T_3-binding sites in rainbow trout. Darling et al., 1980, 1982), using an in vitro approach, characterized specific saturable T_3-binding sites in liver nuclei of coho salmon (Oncorhynchus kisutch) with affinity for T_3 25-fold greater than for thyroxine (T_4).

In fish, thyroid hormones influence osmotic and ionic balance, Ca^{++} homeostasis, nitrogen, lipid and protein metabolism, reproduction and growth (reviewed by Eales, 1979a). A classical effect of TSH-stimulated thyroid activity is marked skin silvering attributed to elevated integumentary guanine content (Premdas and Eales, 1976b). Evidence suggests that thyroid hormones influence salmonid gill Na^+/K^+-ATPase activity (Clarke et al., 1978; Dickhoff et al., 1977, 1978a; Folmar and Dickhoff, 1978, 1979).

In order to investigate fish thyroid function it is frequently necessary to experimentally decrease or increase plasma levels of thyroid hormone thereby altering the occupancy of thyroid hormone receptors in target tissues. It is difficult to render a fish hypothyroid. In most fish thyroid tissue is dispersed in the basibranchial region, and

does not significantly accumulate plasma iodine. Consequently
surgical and radiothyroidectomy are impractical. The
usefulness of inhibitors, such as thiourea and propylthiouracil,
to suppress plasma thyroid hormone levels has been reviewed
(Eales, 1979a). Inhibitors, in general, are not satisfactory
because of the potential for secondary non-thyroidal effects,
mixed success, and toxicity of doses required to alter plasma
thyroid hormone levels. Elevation of plasma T_3 above
endogenous levels by administration of exogenous T_3 is
possible. Of the several methods that exist for elevating
plasma levels of thyroid hormones, the least invasive and
least stressful involves uptake of ambient hormone. This
method has been used on a limited basis for plasma T_4
manipulation but uptake of ambient T_3 by fish is restricted
to one preliminary study based entirely on use of labelled
T_3 (Eales and Collicutt, 1974). In view of the potential
significance of T_3 as an active hormone it is of interest
to determine the reliability of the immersion technique
for modifying fish thyroidal status at the plasma and
intracellular (nuclear) levels.

The specific objectives of this study were to determine
if:

(1) a predictable relationship between ambient and plasma
T_3 exists and if the chronically stable steady-state
can be established for plasma T_3 employing the immersion
technique.

(2)   the properties of T_3 nuclear sites can be studied during immersion and if % occupancy can be predicted.

(3)   site occupancy can be related to physiologic responses such as changes in Na^+/K^+-ATPase activity or integumentary guanine content.

LITERATURE SURVEY

A. INTRODUCTION

The metabolism and action of thyroid hormones in
fish have been reviewed (Eales, 1979a) and are the subject
of active investigation. The trout thyroid consists of
follicles dispersed in the basibranchial region. Thyroxine
(T_4), secreted by the thyroid gland, enters the blood
where it is free or bound to plasma proteins or deiodinated
to T_3. In mammals T_3 may be deiodinated to diiodothyronine
analogues. However, this pathway does not appear to be
prominent in trout. Both hormones are subject to various
degradative and excretory processes the most prominent of
which are glucuronide conjugation and biliary excretion.
T_4 and T_3 enter cells where they may effect their biological
responses.

B. PROTOCOLS FOR THYROID HORMONE ADMINISTRATION TO AQUATIC
   VERTEBRATES.

As yet, no specific review exists on the subject,
nor has an attempt been made to experimentally compare
protocols for the administration of thyroid hormone to
aquatic vertebrates. Non-immersion techniques that have
been used (Eales, 1979a) include:

   (i) injection into the heart or coelom
   (ii) injection through plastic tubing via the anus
        into the gut posterior to the stomach

(iii) surgical implantation of semisolids or osmotic

     pumps into the coelom

(iv) feeding thyroid hormones incorporated into rations.

Injection protocols are inadequate for the chronic

maintenance of plasma thyroid hormone levels. The effect of

a single injection decreases with time due to metabolic

clearance. Regularly repeated injections impart pulses

in plasma hormone levels and induce stress due to frequent

anaesthetization and handling. Surgical trauma is a

consequence of implantation techniques. Osmotic pumps

are expensive. Coelomic dimensions restrict the useable

size of both osmotic pumps and semisolid implants. Trout

are intermittent feeders so no prediction may be made in

the timing and quantity of food ingested. Food that is

not consumed may dissolve and together with excreted feces

may release hormone and metabolites into the water.

Fish and amphibian tadpoles have been immersed in

solutions of thyroid hormone to elevate plasma levels.

Etkin (1935, 1968) immersed frog tadpoles in T_4 and

encountered difficulty due to the precipitation of T_4

in aquaria. Frog tadpoles have also been immersed in

thyroid hormones by Kaltenbach (1982) and Krug et al. (1980).

Brook trout (Salvelinus fontinalis), black bullhead

(Ictalurus melas) and channel catfish (I. punctatus) have

been immersed in T_4 (Eales, 1974). Brook trout have also

been exposed to radioactive T_3 (Eales and Collicutt, 1974).
Dickhoff et al. (1977) immersed coho salmon (Oncorhynchus
kisutch) in solutions of various T_4 concentrations.

No documentation is available describing immersion
of aquatic organisms in carrier T_3.

C. T_3 TRANSPORT INTO CELLS AND NUCLEI

In mammals less than 1% of plasma T_3 is free or not
bound to protein (Rao, 1981). This free T_3 interacts
with a plasma membrane component that mediates T_3 uptake
into the hepatocyte (Rao et al., 1976a, b). Hepatocyte
T_3 uptake is saturable, temperature sensitive, dependent
on thiol groups (Eckel et al., 1979) and is affected by
metabolic inhibitors (Krenning et al., 1978; 1982). Similar
properties have been reported for cultured fibroblasts
(Cheng et al., 1980) and passage and the blood-brain
barrier (Meitus and Pardridge, 1980; Pardridge and Meitus,
1980). T_3 uptake requires ATP and is discrete from T_4
transport (Krenning et al., 1980; 1982). In warm blooded
vertebrates, transport of T_3 from the outside of the plasma
membrane to the nuclear binding site may be inhibited
(Halpern and Hinkle, 1982), whereas no such mechanism is
proposed for cold blooded vertebrates (Rana catesbina)
(Toth and Tabachnick, 1980).

In conclusion, evidence suggests active processes of T_3
transport into the cell.

D. T_3 - BINDING SITES

    1.   Distribution in tissues and species.

High-affinity, low-capacity saturable T_3-binding sites

have been described in a variety of tissues from several

organisms including: mammals, birds, amphibians and fish

(Table 1).   I am unaware of any reports on reptilian

T_3-binding sites.   Specific T_3-binding sites occur in the

rat anterior pituitary (Schadlow et al., 1972) and posterior

pituitary, median eminence and brain (Gordon and Spira,

1975).   Similarity of chromatographic elution patterns on

DEAE-Sephadex columns, sedimentation patterns on 5-20%

sucrose gradients and binding affinity for various thyroid

hormone analogs indicate that brain and liver T_3-binding

sites are identical (Schwartz and Oppenheimer, 1978b).

    2.   Isolation of nuclei and binding sites and

        demonstration of saturable T_3-binding.

Techniques for isolation of nuclei for the investigation

of saturable T_3-binding to nuclear sites follow a common

protocol.   Livers are homogenized in a Potter homogenizer

on ice and homogenates are centrifuged at low speed to

produce a crude nuclear pellet which is purified by

centrifugation through 2.3-2.4 M sucrose (Widnell and Tata,

1964; Blobel and Potter, 1966; Samuels and Tsai, 1973, 1974).

When an in vivo isotopic displacement method is used,

radioactivity of nuclei from animals exposed to radioactive

T_3 ($*T_3$) and nuclei from animals exposed to $*T_3$ and carrier

TABLE 1. Summary of tissue nuclear T_3 binding affinities (Ka) and maximum binding capacities (MBC) for several species and tissues.

Animal and Tissue		in vivo or in vitro	Ka (M^{-1})	MBC	Source
Human	Lymphoctye	in vitro	3.25×10^{10}	40 mol T_3/mg DNA	Tsai and Samuels, 1974
	Neutrophils	in vitro	0.91×10^{10}	4.3 mol $T_3/10^7$ cells	Burke and McGuire, 1978
	Monocytes	in vitro	$1.2-3.9 \times 10^9$	0.11-0.9 pmol T_3/mg protein	Maberly et al., 1982
	Liver	in vitro	1.0×10^9	1.01 pmol T_3/mg DNA	Schuster et al., 1979
	Kidney	in vitro	1.0×10^9	0.356 "	"
	Fetal Lung	in vitro	2.85×10^{10}	250-420 "	Gonzales and Ballard 1981,1982
	Breast cell cancer	in vitro	1.00×10^{10}	——	Burke and McGuire, 1978
Rat	Liver	in vitro	——	1.13 pmol T_3/mg DNA	Schuster et al., 1979
	Liver	in vitro	6.1×10^8	1.7 pmol T_3/nuclei/g liver	Koerner et al. 1974
	Liver	in vivo	——	0.639 pmol T_3/mg DNA	Silva and Larsen, 1978
	Liver	in vitro		3.57 pmol/g liver (= 10400 T_3/cell nucleus)	DeGroot and Strausser, 1974
	Kidney	in vitro	——	0.386 pmol T_3/mg DNA	Schuster et al., 1979
	Kidney	in vivo	——	0.193 "	Silva and Larsen, 1978
	Lung	in vitro	3.93×10^9	0.25 "	Morishige and Geurnsey, 1978
	Pituitary	in vivo	——	1.43 "	Silva and Larsen,1978
	Pituitary	in vivo	5.59×10^9	——	Eberhardt et al., 1976
	Kypothalamus	in vivo	3.50×10^9	——	Eberhardt et al., 1976
	Kypothalamus	in vivo	——	0.15 pmol T_3mg/DNA	Schwartz and Oppenheimer, 1978b
	Cerebral hemisphere	in vivo	6.76×10^9	——	Eberhardt et al., 1976
	Cerebral cortex	in vitro	——	1.08 "	Schwartz and Oppenheimer, 1978b
	Cerebral cortex	in vivo	——	0.395 "	Crantz et al., 1982

Species/Tissue	Condition	Affinity	Binding Capacity	Reference
Cerebral Cortex Synaptosomal fraction	in vitro	3.11×10^{10}	4.7 pmol T_3/mg DNA	Mashio et al., 1982
Cerebrum	in vitro	3.79×10^8	0.324 pmol T_3/mg DNA	Mashio et al., 1982
Rat Telencephalon	in vitro	——	0.30 pmol T_3/mg DNA	Schwartz and Oppenheimer, 1978b
Cerebellum	in vito	——	0.141 "	Crantz et al., 1982
Cerebellum	in vitro	——	0.024 "	Schwartz and Oppenheimer, 1978b
Mouse mammary epith	in vitro	1.0×10^9	——	Bhattacharya and Vonderhaar, 1977
Rabbit cultured lung cells	in vitro	2.0×10^9	——	Lindenberg et al., 1978
Fetal rabbit lung cells	in vitro	——	0.267-0.284 mol T_3/µg DNA	Gonzales and Ballard, 1982
Pig Liver	in vitro	——	1.209 pmol T_3/mg DNA	Wiersinga et al., 1982
Rat Liver	in vitro	$0.65-0.68 \times 10^9$	0.333 "	Wiersinga et al., 1982
Chicken embryo liver	in vitro	$1.04-1.07 \times 10^9$	0.363 pmol T_3/ng protein	Bellabarba and Lehoux, 1981
Chicken (nuclear Extract)	in vitro	2.17×10^9	0.863 "	
Quail liver	in vitro	8.92×10^8	5.68 pmol T_3/mg DNA	Weirigh and McNabb, 1981
Bull frog tadpole tail fin (Rana catesbiana)	in vitro	1×10^9	1500 binding/nucleus sites	Yoshizato et al., 1975
" Liver		1.47×10^9	12300 "	Yoshizato et al., 1975
" Liver	in vitro	0.35 pmol T_3/mg DNA (2700 sites/nucleus)	0.35 pmol T_3/mg DNA (2700 sites/nucleus)	Toth and Tabachnick, 1979
" Liver	in vitro	$k_{T3} = 15 \times 10^9$ $K_{T4} = 2.2 \times 10^9$	12300 T_3 sites nucleus 2300 T_4 sites	Kistler et al., 1975
Toad urinary bladder (Bufo marinus)	in vitro	1.54×10^{10}	6.4 pmol T_3/mg DNA	Geering and Rossier, 1980
Rana catesbiana tadpole liver	in vitro	$k_a T_3 = 6.25 \times 10^{11}$ $k_a T_4 = 2.56 \times 10^{11}$	T_3 MBC = 0.1 ng/mg DNA T_4 MBC = 0.5 ng/mg DNA	Galton, 1980
Rainbow trout liver (Salmo gairdneri)	in vivo	$0.9-1.2 \times 10^8$ (kg liver/mol T_3)	0.43-0.62 pmol T_3 g liver	Van der Kraak and Eales, 1980
Coho salmon liver (Oncorhyncus kisutch)	in vitro	affinity for T_3 25 x greater than for T_4		Darling et al., 1980
"	"	1.03×10^9	127 pmole T_3/g DNA	Darling et al., 1982

T_3 are compared. Alternatively nuclei may be incubated in vitro in *T_3 with various concentrations of carrier T_3 or with increasing amounts of *T_3 (Surks et al., 1975; Groul, 1977). Traditional analysis of these data (Scatchard analysis) may be limited by non-specific binding (Klotz, 1982).

Nuclei or chromatin may be exposed to nuclease digestion to release binding sites from chromatin (Groul, 1980b). Binding sites may be solubilized by KCl extraction (Bernal and DeGroot, 1977; Samuels et al., 1974a; Latham et al., 1976). Protocols for the examination of extracts include DEAE-sephadex chromatography (Silva et al., 1977), affinity chromatography (Latham et al., 1981), sometimes using hydroxylapatite as a support matrix (Groul, 198a), and photo affinity labelling (Pasqual et al., 1982). The use of divalent cations (Rodriquez-Pena and Bernal, 1982b), monodansylcadaverine (Horiuchi et al., 1982) and mercurial and chaotropic agents (Menezes-Ferreira et al., 1982) may induce artifactual results.

3. Characterization of nuclear binding sites.

Saturable nuclear T_3 binding sites have a molecular weight estimated between 50,000 and 70,000 daltons (Torresani and Anselmet, 1978; Groul and Kempner, 1982; Oppenheimer et al., 1974b). Stokes radii of binding sites

released by micrococcal nuclease digestion of chromatin
are 6.5 and 3.3nm respectively for the 6.5s and 3.8s
sedimenting groups (Perlman et al., 1981, 1982). The 6.5s
form is thought to be composed of binding site, chromosomal
protein and DNA fragment. The actual binding site
seminentation coefficient is less than 4s (DeGroot and
Torresani, 1975).

Saturable T_3-binding sites in rats are specific
(Oppenheimer et al., 1972), intimately associated with
chromatin (Surks et al., 1973; Samuels et al., 1982), can
be extracted from the nuclei with slightly alkaline 0.4 M
KCl, are unstable between 25 and 37°C, and are protease-
and pronase-sensitive (DeGroot et al., 1974; 1976a, b). T_3
in incubation media at 4°C enhances binding of sites to
chromatin (Thomopoulas et al., 1974).

There are limitations due to non-specific *T_3 binding
in the analysis of T_3 binding to solubilized sites (Seelig
et al., 1981b). However, the association constant, MBC,
(maximum binding capacity), weight, and affinity for
iodothyronine analogs are similar for intranuclear and
solubilized sites (Aprilletti et al., 1981; Samuels et al.,
1974b; Torresani and DeGroot, 1975; Yoshizato and Nakajima,
1977; Torresani et al., 1978; Burman et al., 1980).
Triiodothyroacetic acid, D-T_3, and L-T_4 bind respectively
with four times, the same, and one fourth the affinity of
T_3 (DeGroot and Torresani, 1975; Surks and Oppenheimer, 1977).

In vitro T_3 binding to isolated nuclei of adult rat liver, cerebral hemisphere and anterior pituitary is saturable and follows Michaelis-Menten kinetics (Eberhardt et al., 1978). Binding is optimal between pH 7.5-8.5 and is Mg^{++}-sensitive. Solubilized sites separated from other chromatin proteins lose their high affinity for T_3 which can be restored if core histones are added to incubation media. Saturable sites are not tightly associated with nucleosomes, the basic subunit of chromatin, but are associated with linker DNA in structurally modified regions of chromatin (Jump et al., 1981; Gardner, 1978). Histones may regulate non-histone chromosomal proteins that regulate gene expression (Coulombe et al., 1979).

  4. Saturable T_3-binding in various physiologic

    states.

  (a) Food deprevation

Starvation depresses MBC (maximum binding capacity) in trout (Van der Kraak and Eales, 1980). Fasted and fed rats have common hepatocyte nuclear ka values ($5.2-6.2 \times 10^8$ M^{-1}) but fasted rats have depressed liver MBC values in comparison to fed rats ($0.3 - 0.46$ nmole T_3/mg DNA) (Burman et al., 1977). Similar results have been found for brains of starved rats (Thrall and Yanagihara, 1982). Depressed MBC, associated with low plasma T_3 levels, and starvation may be a mechanism to protect animals with low caloric reserves from protein depletion (Schussler and Orlando, 1978; Dillman et al., 1978b). In nutritionally deficient rats plasma T_3 does

not influence occupancy, possibly because T_3 entry into the cell and/or nucleus may be inhibited partially (Okamura et al., 1981). Fasting does not affect monocyte MBC in normal and obese people (2.3 and 2.5 fmole T_3/ 100 µg DNA) nor k_d values (2.3 and 2.4 x 10^{-11}M) (Buergi and Larsen, 1982).

(b) Altered plasma glucagon and insulin levels.

Elevated plasma glucagon, coincident with starvation in rats and humans, depresses liver T_3 MBC (Dillman et al., 1978a) and may inhibit the synthesis or degradation of nuclear T_3-binding sites. A significant decrease in rat lung T_3-MBC occurs as a result of steptozotocin-induced diabetes, whereas Ka is not affected (Das and Ganguly, 1981). It is also reported that steptozotocin-induced diabetes does not influence rat liver nuclear T_3-MBC (Las and Surks, 1981).

(c) Hypo- and hyper-thyroidism and the influence of T_3 on MBC, Ka, and binding sites synthesis and degradation

It is suggested that plasma T_3 levels may influence rat liver T_3-MBC. Starvation-induced hypothyroid rats have low serum T_4 and T_3 levels and depressed liver and brain nuclear MBC. Thyroidectomized or propylthiouracil-treated, hypothyroid rats have unaltered Ka and MBC values (Murthy et al., 1978) similar to those in lungs of hypo- and hyperthyroid rats (Ruel et al., 1982).

There is contradictory evidence that experimental manipulation of T_3 concentrations may influence Ka, MBC, and receptor half-life (Inoue et al., 1981; Raaka and Samuels, 1981; Rodigues-Pena and Bernal, 1982a). Nuclear T_3-MBC is depressed in nuclei incubated in vitro with T_3 (Jaffe and Samuels, 1977) and in cultured pituitary cells exposed to T_3 (Samuels et al., 1977). T_3 treatment of rats in vivo and rat tissues in vitro increased MBC (Hamada et al., 1979). It is important to note that chronic low level T_3 treatment of rats has been shown to increase nuclear T_3-MBC (Nakamura et al., 1979).

E. SELECTED PHYSIOLOGIC RESPONSES TO T_3

1. Description of Na^+/K^+-ATPase

T_3 has been reported to have significant influence on Na^+/K^+-ATPase which mediates active transmembrane transport of Na^+ and K^+ ions (Skou, 1965). Na^+/K^+-ATPase is located in the cell membrane, has an affinity for Na^+ on the outside and an affinity for K^+ on the inside of the cell and can be found in all cells with Na^+-and K^+-linked active transport. The enzyme hydrolyzes ATP at a rate dependent on extracellular K^+ and intracellular Na^+ concentrations. Na^+/K^+-ATPase activity has been described in a variety of tissues that include erythrocyte membranes, brain, nerve, kidney, muscle, liver, intestine, electric tissue, parotid gland, frog skin, ciliary body, lens,

retina, thyroid tissue and toad bladder (Skou, 1965).
Purification of the enzyme to a specific activity of 881
μmoles Pi/hr/mg protein (units) consists of the
treatment of the isolated microsomal fraction with
deoxycholate and subsequent fractionation by differential
sucrose gradient centrifugation (Jorgensen and Skou, 1969).
The enzyme is composed of subunits each with a molecular
activity of 1,100 - 12,200 Pi/min (Jorgensen, 1974). The
protein subunits are composed of 2 large and 6 small chains,
have an isoelectric point between 4.7 - 4.9, and the
smaller sialoglycoprotein subunit is 55,000 - 57,000 daltons
and is found in a ratio of 2:3 to the larger subunit. The
large α-subunit is 100,000 daltons and contains the ATP
hydrolysis site while the function of the β-subunit is
unknown. Oubain, a cardiac glycoside, specifically
inhibits the enzyme probably by causing a conformational
change which extends an unusually large distance across
the membrane (Carilli et al., 1982).

2. Influence of T_3 on mammalian Na^+/K^+-ATPase
    activity.

Numerous studies have been undertaken to evaluate T_3
influences of Na^+/K^+-ATPase in mammals (Table 2). In the
overwhelming majority in _in vivo_ and _in vitro_ experiments
T_3 has been found to increase Na^+/K^+-ATPase activity by
induction of this enzyme.

TABLE 2. Thyroid hormone influence on Na^+/K^+-ATPase activity and oubain sensitive oxygen consumption $[QO_{2(t)}]$.

Protocol	Observations	Source
Euthyroid (Eu) rats injected with T_3 then liver and diaphragm slides assayed for enzyme activities	a) $[QO_{2(t)}]$ increases ($\Uparrow$) by 90 % and 60%. b) $Na^+ : K^+$ concentration ($[\ ]$) gradients similarly $\Uparrow$.	Ismail-Beigi and Edelman, 1970
Single injection of T_3 into mature Eu rats	a) $\Uparrow [QO_{2(t)}]$ in liver and kidney but not brain b) 60% $\Uparrow$ liver Na^+/K^+-ATPase but no affect on Mg^{++}-ATPase	Ismail-Beigi and Edelman, 1971
T_3 injected (50 µg/100g BW) on alternate days into Eu and thyroidectomized (TX) rats	a) $\Uparrow Na^+/K^+$-ATPase and maintained from 0.85 to 1.3 µmoles Pi/hr/mg protein) (units). b) Single T_3 dose has a smiliar but short-lived response c) Greater $\Uparrow$ (91%) in units in TX than Eu (69%) rats.	Ismail-Beigi and Edelman, 1974a Ismail-Beigi and Edelman, 1974b
Single T_3-injection into rats.	a) $[QO_{2(t)}]$ correlated with Na^+/K^+-ATPase activity by virtue of concommitant T_3 mediated alteration in electrolyte distribution in rat tissues. ie. No affect on plasma Na^+, K^+, or Cl^- but $\Downarrow$ intracellular Na^+, and $\Uparrow$ intracellular K^+ of liver, diaphragm and heart.	Ismail-Beigi and Edelman, 1973

Incubate rat small intestine mucosal cells with T_3	a) 84% ↑ $[QO_{2(t)}]$ from the Eu to the hyperthyroid (hyper) state	Liberman et al., 1976
T_3-injection into EU and Tx rats	a) 75% ↑ in skeletal muscle Na^+/K^+-ATPase and $[QO_{2(t)}]$ b) Correlation between dose and response.	Asano et al., 1976
Single T_3-injection (50 μg/100g BW).	a) 45% ↑ in renal cortical microsomal Na^+/K^+-ATPase 48 hr postinjection b) No affect on meduilary or papillary Na^+/K^+-ATPase or Mg^{++}-ATPase	Lo et al., 1976
Three doses T_3 (50 μg/100g BW)	a) 1st response ↑ 134% Vmax 2nd response ↑ 79% Vmax 3rd response ↑ 46% Vmax b) No change in ka	
Injection of [$-^{32}P$]-ATP and [^3H]- oubain	a) Enzyme activity is linearly related to Vmax and number of enzyme sites.	
Single dose T_3 (50 μg/100g BW)	a) ↑ synthesis of 92000 MW enzyme subunit, 44% by 8 hr, 61% by 20 hr. b) No affect on degradation rate	Lo and Edelman, 1976
Single dose of T_3 (50 μg/100 & BW) into hypothyroid (hypo) rats	a) ↑ in heart Na^+/K^+-ATPase, cardiac output, and $[QO_{2(t)}]$ b) ↑ enzyme activity due to numbers of sites	Phillipson and Edelman, 1977a Phillipson and Ebelman, 1977b
Incubate 1° monolayer rat hepatocytes with 2×10^{-7} M T_3	a) ↑ Plasma membrane $[(QO_{2(t)}]$ at 24 hr then by >50% between 72-96 hr. b) ↑ Na^+/K^+-ATPase and α-GPD in parallel c) 4 hr exposure to T_3 is sufficient	Ismail-Beigi et al., 1979

In *vitro* incubation of epithelial cells of stripped rat jejeunal mucosae with T_3.	a) $\uparrow$ [$QO_{2(t)}$] Na^+/K^+-ATPase
	  57% hypo Eu
	  29% Eu Hyper
		Liberman *et al*., 1979

Incubation of renal cortical tissue in T_3, and [^3H] - or [^{35}S] - Methionine	a) [^3H]-MET: Na^+/K^+-ATPase activity
	  $\uparrow$ 35% by 8 hr
	  $\uparrow$ 32% by 22 hr
	  [^{35}S]-MET: Na^+/K^+-ATPase activity
	  $\uparrow$ 49% by 8 hr
	  $\uparrow$ 58% by 22 hr
	b) rT_3 has no affect
	c) No T_3 influence on degradation rate of enzyme or plasma Membrane sedimentation properties
		Lo and Lo, 1981

Injection of T_3	a) $\uparrow$ parotid and sublingual salivary gland but not submandibular gland Na^+/K^+-ATPase	Lo *et al*., 1981a, b
Injection of T_3 into hypo-rats	a) $\uparrow$ 50% in Na^+/K^+-ATPase activity in liver and salt-independent bile flow	Layden and Boyer, 1976
T_3 and GH administration	a) $\uparrow$ Na^+/K^+-ATPase activity in brain, liver and kidney	Shimomura et al., 1982
T_3-injection	a) $\uparrow$ enzyme activity in brain, liver, kidney, skeletal muscle b) $\uparrow$ enzyme activity in cultured hepatocytes c) $\uparrow$ enzyme activity in rabbit liver and brown adipose tissue.	Lin and Akera, 1978 Pettine, 1981 Klein *et al*., 1981

3. Influence of T_3 on amphibian Na^+/K^+-ATPase activity.

Na^+/K^+-ATPase activity is intimately associated with ion transport. Specific nuclear T_3-binding sites exist in toad bladder (Bufo marinus) and it has been shown (Gerring and Rossier, 1981; Rossier et al., 1978) that T_3 inhibits aldosterone-dependent Na^+ transport. Aldosterone promotes Na^+ reabsorption in a variety of epithelia including toad bladder. T_3 antagonism of aldosterone action is evident in rat kidney. Matty and Green (1964) and Taylor and Barker (1967) describe similar influences on ion movement in toad bladder.

4. Na^+/K^+-ATPase in fish and thyroid hormone influence

It is likely that fish and mammalian Na^+/K^+-ATPase are identical. Kamiya and Utida (1968) report that eel (Anguilla japonica) gill Na^+/K^+-ATPase has characteristics similar to those of mammalian Na^+/K^+-ATPase. Chinook salmon (Oncorhynchus tshawytscha) gill Na^+/K^+-ATPase activity is maximal when assay media contain 240 mM NaCl, 120 mM KCl, 20 mM $MgCl_2$, and 10 mM Na_2 ATP at pH 7.2 (Johnson et al., 1977). Oubain specifically inhibits Na^+/K^+-ATPase from gills of coho salmon (O. kisutch) (Zaugg and McLain, 1970).

The function of gill Na^+/K^+-ATPase differs between freshwater (FW) and marine (SW) fish. In freshwater fish,

water absorbed through the gill is excreted as a dilute urine. Gill chloride cell Na^+/K^+-ATPase of FW fish functions in ion capture from the water (Johnson, 1973). In SW, gill Na^+/K^+-ATPase functions primarily to excrete ions to maintain hydromineral balance. Presently it is not clear how Na^+/K^+-ATPase may reverse its effects in a single fish, an anadromous salmonid for example, over a period of time with changed ambient salinity.

Gill Na^+/K^+-ATPase activity varies with ambient salinity. Plasma sodium increases with ambient sodium concentration in eels (A. rostrata) (Butler and Carmichael, 1972). Evans and Mallery (1975) report that FW fat sleepers (Dormitator maculatus) when exposed to 50% SW then to 100% SW show rapidly increased gill protein content followed by activation of Na^+/K^+-ATPase. Gill Na^+/K^+-ATPase activity of the japanese eel (A. japonica) increases during adaptation to SW slowly for the first five days then more rapidly in the succeeding two days (Kamiya and Utida, 1968). Not all FW fish adapting to SW show an increase in gill Na^+/K^+-ATPase activity. Gill enzyme activity of FW flounders (Platychthys flesus) and eels A. anguilla) were constant when fish were exposed to SW (Kirschner, 1969). In general, gill Na^+/K^+-ATPase activities of marine species are greater than those of FW fish. In euryhaline fish the SW adapted groups have greater activities than FW groups (Kamiya and Utida, 1969; Jampol and Epstein, 1970).

Temperature and parr-smolt transformation may be factors influencing increased gill Na^+/K^+-ATPase activity in salmonids (Adams et al., 1973; Zaugg et al., 1972; McCarty and Houston, 1977). Salmonids hatch in FW and in the process of maturation, parr-smolt transformation, migrate to relatively colder SW. A direct relationship between seaward migration of steelhead (S. gairdneri) and gill Na^+/K^+-ATPase activity has been described (Zaugg and Wagner, 1973). Parr-smolt transformation and increased gill enzyme activity will occur only below a critical temperature that varies among salmonid species (Zaugg and McLain, 1976). The critical temperature for steelhead, for example, is $11.3^{\circ}C$ (Adams et al., 1975). Gill Na^+/K^+-ATPase activity, in steelhead undergoing parr-smolt transformation, approximately doubles at the beginning of March at water temperatures between 6.5 and $10^{\circ}C$ (Adams et al., 1973). With the low water temperatures of October, gill Na^+/K^+-ATPase increases in chinook salmon (O. tshawytscha) when they first migrate to SW (Ewing et al., 1979). Na^+/K^+-ATPase activities increased in gill and decreased in kidney of Atlantic salmon (S. salar) during parr-smolt transformation between March and July under hatchery conditions (McCartney, 1976). Retention of migratory chinook in FW results in decreased enzyme activity and regression to parr-like appearance (Zaugg and McLain, 1972; Giles and Vanstone, 1976; Folmar and Dickhoff, 1978). Similar observations have been made for rainbow trout (Zaugg and Wagner, 1973)

and Atlantic Salmon (Saunders and Henderson, 1978).

There is a positive correlation between plasma T_4 levels, adaptation to SW and gill Na^+/K^+-ATPase activity (Clarke et al., 1978). Immersion of FW coho smolt in T_4 (0.01, 0.1 and 1.0 µg/ml) increases plasma T_4 but only lower doses increase gill enzyme activity (Dickhoff et al., 1977). Plasma thyroid hormone concentration and parr-smolt transformation occur concurrently (Dickhoff et al., 1978a, b). T_4 involvement in coho smolting and associated Na^+/K^+-ATPase activity is postulated (Folmar and Dickhoff, 1979). Sower and Schreck (1983) described a relationship between plasma T_3 and smolting in coho salmon.

Fish Na^+/K^+-ATPase is likely similar to the mammalian enzyme and may be influenced by T_4 and/or T_3. Photoperiod, but more so temperature and salinity, are probably involved in the initiation of salmonid parr-smolt transformation. Transformation is accompanied by increased plasma thyroid hormone levels and gill Na^+/K^+-ATPase activity. In summary increased gill Na^+/K^+-ATPase activity may be a consequence of elevated plasma thyroid hormone levels induced by environmental factors. Elevated gill Na^+/K^+-ATPase activity may contribute to salmonid smolt salt-water tolerance.

5. Thyroid hormones and integumentary guanine

   in fish

Guanine, an integumentary purine, is the predominant factor contributing to epidermal silveriness of fish (Peschen, 1939; Sumner, 1944). More guanine is contained in epidermal than in scales. Guanase has been used to identify guanine in the epidermal (Hitchings and Falco, 1944) and guanine crystals have been isolated from rainbow trout epidermal (Robertson, 1948). In addition to guanine, hypoxanthine has been identified in a variety of fish (Zeigler-Gunder, 1956a, b) including: the parr and smolt of coho salmon (Markert and Vanstone, 1966); Atlantic salmon (Johnson and Eales, 1967); and brown trout (S. trutta) (Massey and Smith, 1968). Eales (1969) described the contribution of guanine and hypoxanthine to epidermal silveriness in: channel catfish (I. punctatus), burbot (Lota lota), brook stickleback (Culea inconstans),pike (Esox lucius), walleye (Stizostedion vitreum), sauger (S. canadense), goldfish (Carassius auratus), trout-perch (Percopsis omiscomaycus) and brook trout (Salvelinus fontinalis). Visible "silvering" of fish occurs in smolting salmonid fish as a result of deposited guanine crystals in the iridiophores which obscure melanophores (Landgrebe, 1941; Robertson, 1949; LaRoche and Leblond, 1952).

Increased thyroid activity, measured histologically, accompanies elevated integumentary guanine deposition (Hoar, 1939). Increased thyroid activity occurs prior to the loss of parr marks in four species of Pacific salmon (Baggerman, 1960) and rainbow trout (Robertson, 1948). Dermal silvering has been induced in Atlantic salmon (Landgrebe, 1941), and rainbow trout (Robertson, 1948) with thyroid or pituitary extract, and in brown trout ($\underline{S}$. $\underline{trutta}$) with purified TSH (Smith, 1956). Hypophysectomized mollys ($\underline{Molienesia}$ $\underline{formosa}$) have reduced thyroid activity and appear dull, while T_4 treatment restores the normal silvery state (Ball $\underline{et}$ $\underline{al}$., 1963). Prolonged T_4 treatment increases ^{14}C-glycine incorporation into newly-synthesized guanine (Matty and Sheltawy, 1967). T_4 modifies guanine production (Hoar and Bell, 1950; Hoar $\underline{et}$ $\underline{al}$., 1951). TSH treatment of salmonids ($\underline{S}$. $\underline{gairdneri}$) stimulates integumentary deposition of guanine (Premdas and Eales, 1976b).

MATERIALS AND METHODS

A. EXPERIMENTAL ANIMALS

Rainbow trout (Salmo gairdneri) of Nisqually and domestic Sundalsora stock, less than 1 year old, were obtained from the Federal Fish Hatchery, Balmoral, Manitoba and kept in 2.3-kl fibreglass tanks with flowing 11°C dechlorinated Winnipeg City drinking water. Fish were maintained on Ewos trout pellets (Astra Chemcials Ltd., Mississauga, Ontario) at a ration of 1-2% of body weight (BW) daily and kept under a 12-hr photoperiod. Prior to experimental use, fish were sorted according to size either by visual estimation or by weighing fish lightly-anaesthetized in tricaine methane sulfonate (MS222, Syndell Laboratories Ltd., Vancouver; 0.08 g/ℓ). Fish were tranferred to 175-ℓ fibreglass tanks and allowed to acclimate for 2-3 days under similar conditions prior to the start of an experiment. All experimental tanks were kept in a controlled-environment room (9.8-12.2°C; 12 hr photoperiod).

B. IMMERSION TECHNIQUE

For a given experiment a stock solution of 3,5,3'-triiodo-L-thyronine (T_3) (Sigma) was prepared by dissolving the required weight of the anhydrous sodium salt of T_3 in 0.1 N NaOH, or methanolic ammonia (99:1 v/v)

and then in 0.1 N NaOH. An appropriate volume of stock
solution, always less than 25 ml, was dispensed by an
automatic pipette into static aerated tanks containing
150 l of water to yield the desired ambient T_3 concentration
(estimated as free acid). Tank water was renewed without
undue disturbance to fish by draining half the water, and
then adding the required amount of water and T_3 to
reinstate the volume and ambient T_3 concentration. Tanks
containing control fish were similarly treated with
identical volumes of 0.1 N NaOH but containing no T_3.

C. PLASMA SAMPLING

After immersion for a selected time regime for each
experiment, fish were netted, anaesthetized and blood samples
(400-450 µl) taken from the caudal vessels using a 27-g
1½-inch non-heparinized hypodermic needle mounted on a
1.0-ml heparinized tuberculin syringe. Blood samples were
transferred to 1.0-ml polystyrene microcentrifuge tubes
(Fisher) and centrifuged at approximately 15000 g for
3 minutes in an International Microcapillary Centrifuge,
model MB (International Equipment Company, Needham Hts.,
Mass.). Plasma was aspirated by Pasteur pipette and
transferred into 1.0-ml conical bottomed polystyrene
sample vials (Fisher), capped with parafilm and stored
at $-20^{\circ}C$ for later T_3 analysis by radioimmunoassay.

D. T_3 - RADIOIMMUNOASSAY (T_3 - RIA)

Plasma T_3 concentrations were determined by radioimmunoassay (Brown and Eales, 1977). Barbital buffer consisted of 75 mM sodium barbital at pH 8.6. [^{125}I] T_3 (*T_3) (phenolically labelled, initial specific activity 550 mCi/mg; Industrial Nuclear Co., St. Louis) was diluted with 0.1 N NaOH to yield 5000-7000 counts per minute (cpm) per 100 µl. The level of radioactivity between 15 and 80 KEV was determined by counting samples in a well type Beckman Gamma 8000 gamma counter (Beckman Instruments Inc., Irvine, California) containing a NaI crystal activated with thallium iodide. Samples were counted for either 10 min or 2σ% error at approximately 76% efficiency.

Miniature G-25 fine Sephadex chromatography columns with a bed height of approximately 2 cm in disposable 5-ml syringes were normally stored with 0.1 N NaOH. Columns were drained and bottoms recapped, and all reagents were equilibrated to room temperature prior to use. One hundred µl of *T_3 was pipetted onto the top of the column followed by 100 µl of standard or sample, swirled and bottoms uncapped and the mixture drained into the column. A standard stock solution of T_3 was prepared by dissolving the monohydrated sodium salt of T_3 in 0.1 N NaOH. Standards (0 to 2000 ng%) were prepared by dilution with 0.1 N NaOH. In 0.1 N NaOH T_3 is not protein bound and binds to Sephadex.

A 3.0 ml barbital elution washed off plasma proteins and
iodide. Randomly chosen columns had this fraction
collected and counted for radioactivity for later
calculations. Counting tubes were placed under the columns
and 1 ml of T_3 antibody (Calchemical Lab. Supplies Ltd.,
Calgary, Alberta) diluted 1:22000 with barbital buffer
(yielding approximately 50% binding) was pipetted onto
the columns and allowed to incubate. After 90 to 120
minutes a volume of 2 ml of barbital buffer was applied
to the column to elute the antibody bound fraction. T_3
standard curves and calculation of sample T_3 concentrations
were made by interpolation by performing "probit" or Logit
calculations (Appendix 1). T_3 values in samples were
derived from the regression equation of the linear
relationship between "probit" values of standards and
the natural logarithm of standard concentration. Columns
were regenerated by sequentially passing through 10 ml
(DD) H_2O (distilled and deionized), 8 ml human plasma
diluted 1:9 (v/v) with barbital buffer, 10 ml H_2O and
8 ml 0.1 N NaOH.

E. BINDING OF T_3 TO NUCLEAR SITES.

    1. T_3 injection and removal of organs.

    The routine protocol involved immersing fish in a
concentration of ambient T_3 for a designated period,
removing a blood sample and then injecting the anaesthetized

fish with *T_3. Fish were injected intraperitoneally, anterior to the pelvic fins, with [^{125}I]T_3 (initial specific activity 500 mCi/mg, Industrial Nuclear Co., St. Louis) in 20 µl of 50% (v/v) aqueous propylene glycol using a 30-g, ½-inch needle attached to a 1.0-ml tuberculin syringe mounted in a repeating dispenser (PB-600-1, Hamilton Company, Reno, Nevada); thereafter fish were returned to their tanks. At selected times fish were netted, anaesthetized, weighed and blood removed from the caudal vessels, anterior to the previous puncture site, with a 21-g, 1½-inch heparinized needle mounted on a 3.0 ml heparinized syringe. Plasma was obtained and stored and the fish killed by concussion.

A ventral median incision was made from the anus to the isthmus of the gills. The gall bladder was removed and placed into a 16- x 125-mm borosilicate glass disposable culture tube (Fisher) with 0.1 N NaOH to contain a total volume of 4.0 ml.

The liver was removed, blotted dry and weighed to the nearest 0.01 g in a 50-ml disposable polystyrene microbeaker (Fisher) to which was added 4 volumes of ice-cold Buffer A (3 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM spermidine and 10 mM tris, pH 7.7 and 0.32 M sucrose) (Van der Kraak and Eales, 1980) and stored on ice.

2. Isolation of Nuclei

All procedures involved in the isolation of nuclei were performed at 0°C. Individual livers (approximately 1.0 g) were homogenized by 4 strokes of a hand-held glass mortar with a tight-fitting motorized teflon pestle at 750 RPM for 1 min (Tri-R Stir-R model K43, Tri-R Instruments Inc., Rockville Centre, N.Y.). The homogenate was transferred to a glass 13-ml graduated cylinder and the mortar and pestle flushed with 4 volumes of buffer A, pooled with the homogenate and the volume recorded. The cylinder was parafilm capped and inverted several times, contents poured into a 13-ml Autoclear polycarbonate centrifuge tube (IEC) and duplicate 100-μl aliquots were removed, placed into glass tubes with 3.9 ml 0.1 N NaOH and counted to estimate total liver radioactivity. The homogenate was centrifuged at 700 g (10 min) in a refrigerated centrifuge (Model B-20, Rotor No. 846, International Equipment Co., Needham Hts., Mass.) to obtain a crude nuclear pellet. The supernatant was decanted and discarded and the pellet was resuspended with 8 volumes of Buffer B (Buffer A with 2.3 M sucrose instead of 0.32 M sucrose) using a loose-fitting, motorized teflon pestle. The resuspended pellet was centrifuged at 35000 g (60 min), the floating nuclear debris scraped off the surface and the supernatant decanted

and discarded. The greyish white pellet of nuclei was resuspended with 2.5 ml of Buffer A using a loose-fitting motorized teflon pestle and put into a glass counting tube. A 2.5 ml aliquot of Buffer A was used to flush the tube and pestle and pooled with the resuspended pellet. The final volume was adjusted to 5.0 ml with Buffer A. Nuclei were counted for radioactivity and frozen. In a trial preparation the nuclear pellet was examined under interference microscopy and observed to contain intact nuclei without cells or cellular debris.

3. DNA Measurement

(a) DNA Extraction

According to Widnell and Tata (1964), 8 ml of ice-cold 0.2 N $HClO_4$ was pipetted into 1-ml of resuspended nuclei in a 13-ml polycarbonate centrifuge tube, put on ice and then centrifuged at 10000 g (0-4°C; 10 min). The resulting supernatant was decanted and discarded and the procedure repeated with the pellet resuspended using a loose-fitting motorized teflon pestle. Four ml of 0.5 N $HClO_4$ was pipetted onto the pellet to be resuspended. The suspension was heated for 15 min at 75°C in a water bath. Immediately afterwards the samples were chilled on ice and recentrifuged. The hydrolyzed DNA was decanted and saved. The procedure was repeated, the extract pooled and adjusted to 8 ml with 0.5 N $HClO_4$.

(b)   Development and Determination

DNA standard solutions (0, 6.25, 12.5, 25, 50, 100

µg DNA/ml) were prepared by the serial dilution of a DNA

stock solution. The DNA stock solution was made by

dissolving calf thymus DNA (0.4 mg DNA/ml 0.005 M NaOH),

then heating twice for 15 min at $75^{O}C$. A volume of

0.5 N $HClO_4$ was used to dilute this stock solution.

One-ml aliquots of the DNA extract or standard were

vortexed in a glass tube with 2.0 ml of fresh diphenylamine

reagent (Burton, 1956) (1.5 g diphenylamine, 100 ml,

glacial acetic acid, 1.5 ml of concentrated sulphuric acid

and 8.12 mg aqueous acetaldehyde). The mixture was

parafilm-capped and incubated for 20 hr in darkness at

$30^{O}C$ in a shaking metabolic water bath. DNA was measured

photometrically by absorbance at 595 mµ in a Perkin-Elmer:

Coleman 124 double beam spectrophotometer. Absorbance

was plotted as a function of DNA concentration of standards

and sample concentrations were calculated from the

regression equation of the linear relationship.

4.   Calculation of the equilibrum constant (K),

maximum binding capacity (MBC) and occupancy

of saturable T_3-binding sites of liver nuclei

Ligand binding to saturable and non-saturable sites

may be distinguished by Scatchard analysis (Rodbard, 1973).

The present Scatchard analysis is the plot of the ratio

of bound nuclear radioactivity to free liver radioactivity

(B/F) as a function of saturably bound nuclear T_3

(B) (moles/kg liver). In the present study B and F were

calculated as follows:

$$\underset{\text{(moles } T_3/\text{kg)}}{B =} \frac{\text{liver nuclear bound radioactivity (cpm)}}{\text{plasma specific radioactivity (cpm/mole } T_3)} \times \frac{1}{\text{liver wt (kg)}}$$

where,

$$\underset{\text{(cpm/mole } T_3)}{\underset{\text{radioactivity}}{\text{plasma specific}}} = \frac{{}^*T_3 \text{ (cpm/100 } \mu\text{l plasma)}}{T_3 \text{ (moles/100 } \mu\text{l plasma)}}$$

and,

$$\underset{\text{(moles } T_3/\text{kg)}}{\text{free liver } T_3 \text{ "F"}} = \text{T-B}$$

where,

$$\underset{\text{(moles } T_3/\text{kg)}}{\text{total liver } T_3 \text{ "T"}} = \frac{\text{liver radioactivity (cpm)}}{\text{plasma specific radioactivity (cpm/mole } T_3)} \times \frac{1}{\text{liver wt (kg)}}$$

Saturable T_3 binding is defined as the phase of the Scatchard

curve with negative slope. Non-saturable binding, observed at

high carrier doses, is represented by a theoretically horizontal

assymptote. Subtraction of the average B/F corresponding

to non-saturable binding from observed B/F values of

saturable binding defines the corrected B/F.

Alternately, in the Wilkinson plot (Appendix 2) the reciprocals

of the corrected B/F values (F/B) are plotted as a function

of F. Linear regression of the plot of F/B as a function

of F describes a best-fit straight line where maximum

binding capacity (MBC) is the reciprocal of the slope.

The equilibrium constant (K) is the slope divided by the

ordinate intercept. MBC (moles T_3/kg) represents the

amount of saturably bound nuclear T_3 in a fully occupied

population of saturable binding sites. Saturably bound

nuclear T_3 (moles T_3/kg) is determined by multiplying

corrected B/F by T (moles T_3/kg). The proportion of the

total number of saturable sites (MBC) bound with T_3 is

defined as occupancy.

The assumptions used in these calculations were:

1. T_3 is not changed or degraded.

2. At the time of sampling T_3 metabolism was constant.

3. Isotopic equilibrium in the liver exists at the time

    T_3 determinations were made.

4. Free hormone represents all liver T_3 that is not

    saturably nuclear bound.

F. SEPARATION OF LABELED IODOMATERIALS IN PLASMA

Miniature G-25 (fine) Sephadex columns with a bed

height of 3.5 cm were used to chromatographically separate

iodoprotein, iodide and iodothyronine (T_3) fractions from

plasma of fish injected with $*T_3$. Columns stored with

0.1 N NaOH were allowed to drain after which the bottoms

were capped. On top of the column 100 μl of 0.1 N NaOH

and 100 μl of radioactive plasma were pipetted, swirled

and allowed to drain into the column after the bottoms

were uncapped. Two elutions of 2.25 ml barbital buffer

were collected. These comprised the iodoprotein

(non-hormonal covalently-linked iodine) and iodide,

including radioiodide ($*I^-$) fractions respectively.

Thereafter two 4-ml elutions of human plasma diluted 1:9

(v/v) with barbital buffer were collected.

It is assumed that the radioactivity in these fractions

represented $*T_3$ (Higgs and Eales, 1971). Columns were

regenerated by sequential elutions of 10 ml water and 10 ml

0.1 N NaOH.

G. DETERMINATION OF Na^+/K^+-ATPase ACTIVITY

1. Isolation of microsome-associated enzyme.

Isolation of the microsome-associated Na^+/K^+-ATPase

(E.C. 3.6.6.1) was accomplished using a procedure modified

from Giles and Vanstone (1976). Fish were anaesthetized,

blood removed for later T_3 analysis and killed by

concussion. Approximately 1.0-g portions of gill filaments

or the liver were weighed and placed in 5.0 ml of

homogenization medium (250 mM sucrose, 10 mM imidazole

and 2.0 mM disodiumethylenediaminetetraacetate (EDTA),

pH 7.4) in a 50-ml polystyrene disposable microbeaker
(Fisher) and held on ice. The sample of gill filaments
or liver was blotted and reweighed and placed into a glass
mortar with 5 volumes of ice-cold homogenization medium.
All subsequent steps were carried out between 0 and 2^{O}C.
Tissues were homogenized with 7 strokes of a tight-fitting
motorized teflon pestle at 750 RPM. The homogenate was
transferred to a 13-ml polycarbonate centrifuge tube and the
5 volumes of homogenization medium which were used to
flush the mortar and pestle were pooled with the homogenate.
The homogenate was mixed by repeated inversion and
centrifuged at 1000 g (10 min) and 2000 g (10 min). The
supernatant was aspirated and transferred into a similar
centrifuge tube and centrifuged at 10000 g (30 min). The
supernatant was aspirated and transferred into another
centrifuge tube and centrifuged at 35000 g (60 min) to
obtain the microsomal pellet which was resuspended with
a loose-fitting motorized teflon pestle in 5.0 ml of
resuspension medium (homogenization medium with 0.1%
(w/v) sodium deoxycholate).

2. Protein determinations

Protein determinations were made by pipetting
5.0 ml of BioRad reagent (BioRad Laboratories,
Mississauga, Ontario) into 100 µl of sample

or standard in a glass tube and mixing by inversion. The
Bio-Rad protein assay is based on the absorbance of
Coomassie Brilliant Blue G-250 shifting from 465 to 595 mμ
in an acid solution when binding to protein occurs (Sedmak
and Grossberg, 1977). Absorbance was measured in a Perkin
Elmer: Coleman 124 double beam spectrophotometer. A set
of protein standards (0, 45, 90, 180, 360, 720, μg/ml) was
prepared by dissolving bovine serum albumin in 0.7% NaCl
with 0.001 g sodium azide to prevent bacterial degradation.
Protein concentrations were determined from the regression
equation derived from the linear relationship between A_{595}
and protein concentration.

3. Enzyme assay

Enzyme activity was based on production of inorganic
phosphate (Pi) from substrate Na_2 ATP. The Na^+/K^+-ATPase
activity (oubain sensitive) was distinguished from the
total phosphatase activity by comparing Pi production with
or without the presence of oubain.

In all cases freshly prepared, resuspended microsomes
were used in enzyme assays. Preliminary investigation showed
that the Na^+/K^+-ATPase activity of resuspended microsomes,
frozen then thawed, was below that of fresh preparations
(Appendix 3).

After 5 min heating at 37°C, 200 μl of microsomal
suspension, diluted to contain 30-40 μg protein, was
pipetted into 1.0 ml of incubation medium (30 mM tris,

5 mM Na$_2$ ATP (Sigma), 5 mM MgCl$_2$, 90 mM NaCl and 20 mM KCl, with or without 4 mM oubain, pH 7.7) in a glass tube and incubated at 37°C in a shaking metabolic water bath (125 RPM). After 20 min the reaction was stopped with 500 µl of ice-cold 17.5% (w/v) trichloroacetic acid, the tube put on ice for 5 to 10 min, and centrifuged at room temperature at 3500 g (10 min) to sediment suspended protein.

Inorganic phosphate was determined photometrically (Fiske and Subbarow, 1925; Hawk et al., 1954). Phosphate standards were prepared by dissolving 0.351 g pure dry monopotassium phosphate (KH$_2$PO$_4$) in 10 ml 10 N H$_2$SO$_4$ and diluted with distilled deionized water to a final volume of 1 ℓ to yield 80 µg/ml inorganic phosphate (Pi). From the phosphate stock solution a set of standards (0, 1.25, 2.5, 5.0, 10, 20, 40, µg/ml) was established by serial dilutions. An indefinitely stable solution of ammonium molybdate was prepared by dissolving 2.5 g ammonium molybdate in 20 ml distilled deionized water, adding 30 ml of 10 N H$_2$SO$_4$ and diluting with water to 1 l. The Fiske and Subbarow Reducing Agent (Sigma) was prepared by dissolving 3.9683 g in 25 ml water and stored in the refrigerator. One ml of standard, or sample, was pipetted into a glass tube containing 200 µl of molybdate agent, after which 80 µl of reducing agent and 720 µl of distilled deionized water were added. The mixture was immediately vortexed and

incubated at room temperature. After 15 min, absorbance

at 690 mµ was measured. The amount of inorganic phosphate

present was determined from the regression equation derived

from the linear relationship between A_{690} and Pi concentration.

Enzyme activity was expressed as the difference in µmoles

inorganic phosphate produced produced per hour per mg

protein between incubation media that did or did not contain

oubain.

H.   EVALUATION OF GUANINE CONTENT OF INTEGUMENT

Integumentary guanine concentrations were evaluated

according to Chua and Eales (1971). Strips of integument

(2 cm x 4 cm) immediately below the dorsal fin with the

lateral line bisecting the short axis of the strip were

removed from both sides of frozen fish. Particular care

was taken to avoid removing underlying musculature along

with the integument. Strips were thinly cut, put into

glass tubes with 4 ml of 1 N HCl, parafilm capped and

shaken overnight in a metabolic incubator at 125 RPM.

Tubes were vortexed and contents poured into 13-ml

centrifuge tubes. Glass tubes were rinsed with two 1-ml

aliquots of 1 N HCl and pooled in the centrifuge tubes.

The mixture was centrifuged at 3500 g (15 min), and the

supernatant decanted into 10 ml graduated cylinders.

Integument was rinsed with two 1-ml aliquots of 1 N HCl,

pooled, and the final volume recorded. Sample aliquots

of 100 μl were spotted 5 cm longitudinally, 9.5 cm from

the nearest edge and 4 cm from each other on Whatman No. 1

chromatography paper (48 x 48 cm). Descending paper

chromatography was performed using methanol: concentrated

HCl: water   (70:20:10 v/v), in a glass tank

(50 x 20 x 54 cm). Approximately 12 hr later, chromatograms

were removed, air dried, observed under ultraviolet light

(253.57 mμ) and guanine spots outlined (spots nearest the

origin, Chua and Eales (1971)). Areas of paper, 4 x 8 cm,

containing guanine were cut out, cut into smaller pieces

and put into 13-ml polycarbonate centrifuge tubes for

elution overnight (12 hr) with 4 ml of 1 N HCl. Tubes were

centrifuged at 3000 g (10 min), the supernatant decanted

into glass tubes and aliquots read at 220-320 mμ on a Unicam

SP-800 spectrophotometer (Unicam Instruments Limited) using

silica cells. Guanine content (moles/l) of the eluate

was evaluated by dividing A_{248} by the molar extinction

coefficient (11400). Integumentary guanine content was

expressed as μmoles guanine/cm^2.

I. STATISTICAL ANALYSIS

When appropriate a one-way analysis of variance

(ANOVA) (Snedecor and Cochran, 1972) was performed to

determine if experimental treatment of fish groups

elicited significant variations in parameter means. Where

significant variation among group means was detected a
two-sample Student's t-test (Snedecor and Cochran, 1972)
was performed on paired means to determine if there was
a significant difference between them.

RESULTS

A. DEVELOPMENT OF THE IMMERSION TECHNIQUE

The objective was to evaluate immersion as a protocol for chronically raising plasma T_3 concentrations of rainbow trout, predictably, with minimal variation and avoiding undue stress on the fish.

1. Fish were immersed in selected concentrations of T_3 and withdrawn at preset times (1-240 hr)   thereafter to obtain plasma samples to define plasma T_3 concentrations.

Plasma T_3 levels (Fig. 1) increased after 4 hr but were elevated to some extent at 8 hr in all fish including those immersed in 0 µg% T_3. Maximum plasma T_3 levels, for a given ambient concentration, occurred after approximately 24 hr immersion. Plasma T_3 levels changed little between 48 and 96 hr. Fish immersed in 0, 2, 5 or 10 µg/ml T_3 for longer than 120 hr had plasma T_3 levels of 1.7, 3.3, 4.4, and 12.1 ng/ml respectively. Fish immersed as long as 240 hr appeared as healthy and active as they did prior to immersion.

2. In order to determine if $*T_3$ could be administered by immersion fish were immersed in $*T_3$ (5.8×10^{-1} Ci/ml, 23-32%*I) with various stable T_3 concentrations

41

FIGURE 1. Plasma T_3 concentrations of fish immersed
in stable T_3 for various periods. Each
point represents a mean ($\pm$ 2 SEM) of
duplicate samples from four fish.
Body weights and plasma T_3 values given
in Appendix 4.
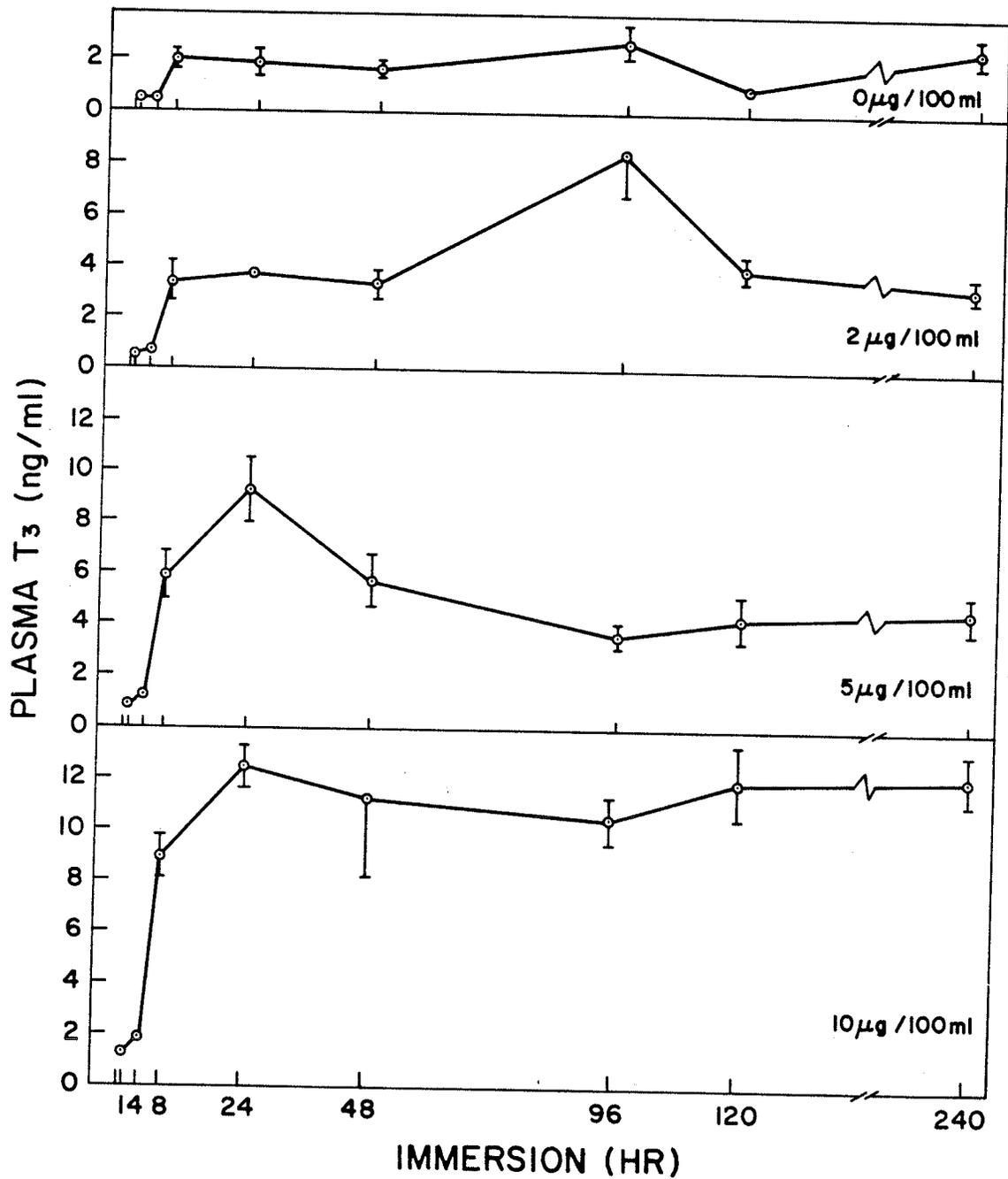
and sampled for plasma, liver and gallbladder radioactivity
at preset times (30 min - 144 hr).

The average fish weight was 50.26 g (SEM = 1.52).
Plasma $*T_3$ (Fig. 2) increased within 15 min of immersion
and maximum levels were found after 4 hr. Fish immersed
for more than 4 hr showed progressively smaller amounts of
plasma $*T_3$. There was no difference in fish plasma
radioactivity between 96 and 144 hr. Liver radioactivity
(Fig. 3) increased to maximal levels between 4 and 8 hr.
In fish exposed to 10 μg/100 ml T_3 maximal liver radioactivity
was maintained for a longer period (24 hr) than in other
ambient T_3 concentrations. After 48 hr liver radioactivity
was lost slowly. The time-course of gall bladder radioactivity
(Fig. 4) differed from that of plasma and liver. Gall bladder
radioactivity increased and decreased gradually, the first
increase in gall bladder radioactivity being detected between 1
and 2 hr. Maximal gall bladder radioactivity was observed
at 24 hr for all T_3 concentrations.

3. Protocols for manipulating plasma stable T_3 levels, and,
plasma and liver radioactivity ($*T_3$) have been established
above, but their application to studying saturable liver
nuclear T_3-binding sites require evaluation. In this
experiment an attempt was made to determine if labeling

43

FIGURE 2. Plasma $*T_3$ in fish immersed for periods

in $*T_3$ with various concentrations of

carrier T_3. Each point represents the

mean of two fish with individual points
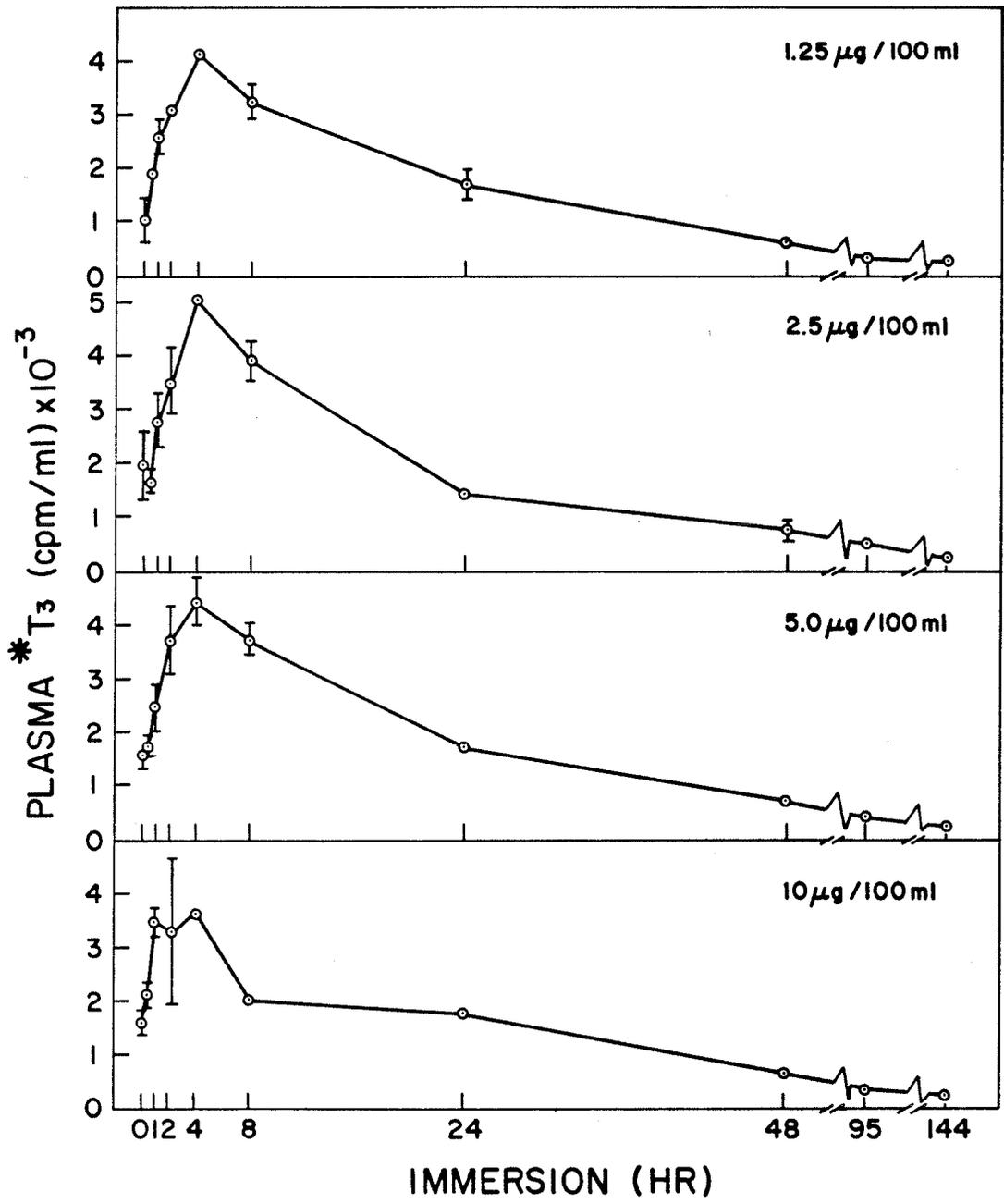
plotted.

FIGURE 3. Total liver $*T_3$ of fish immersed for
periods in $*T_3$ with various concentrations
of carrier T_3. Each point represents
the mean of two fish with individual points
plotted.
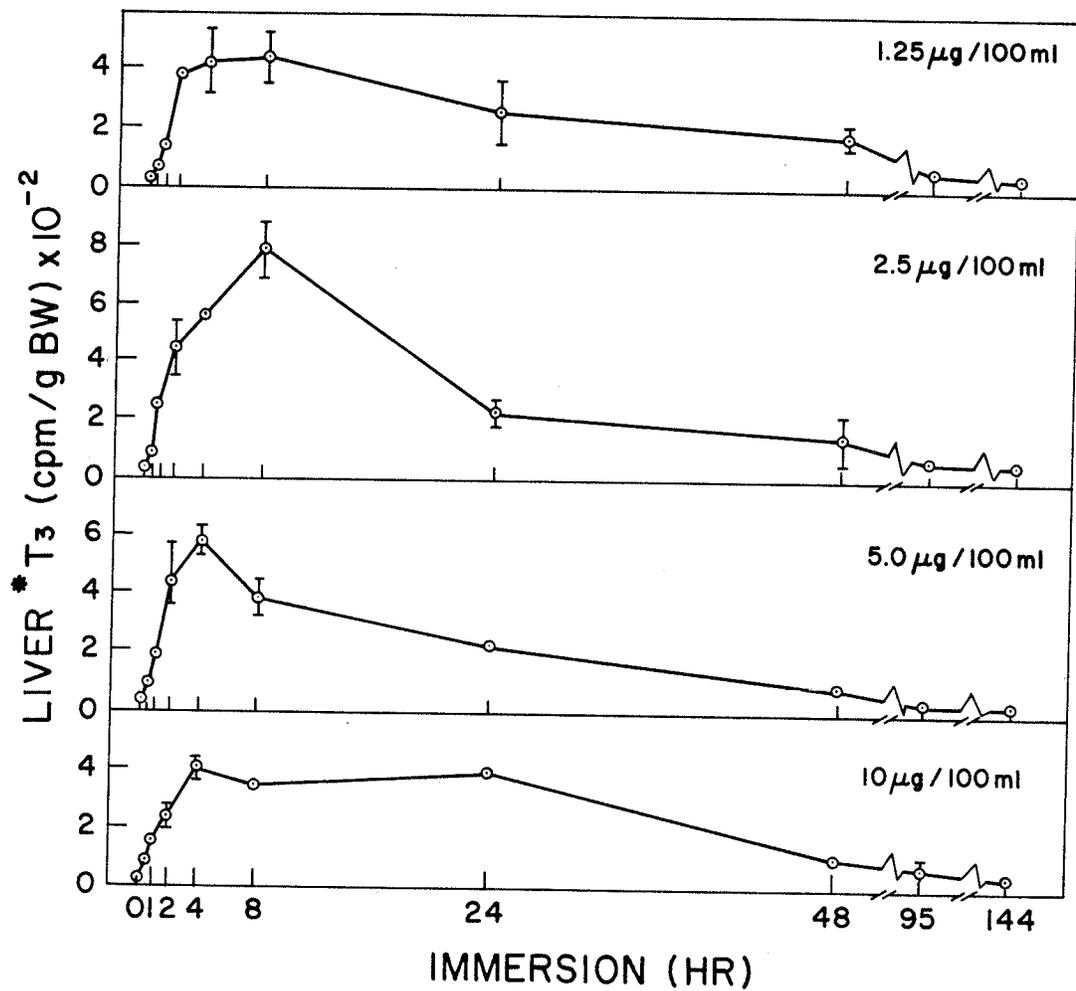
FIGURE 4. Gall bladder $*T_3$ in fish immersed for
periods in $*T_3$ with various concentrations
of carrier T_3. Each point represents the
mean of two fish with individual points
plotted.
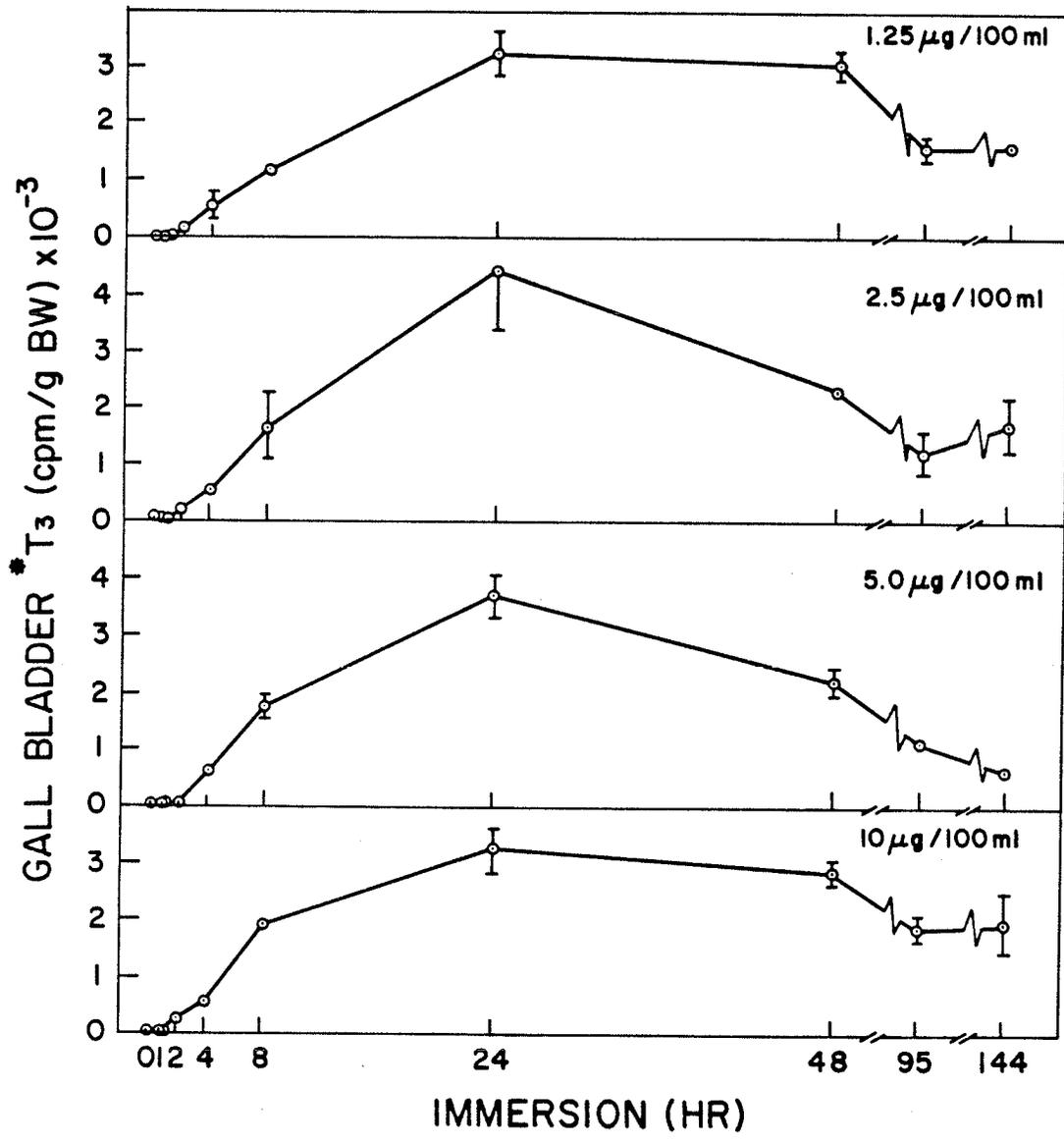
of saturable nuclear sites could be achieved by immersion methods.

Stocks of fish were immersed in 150-ℓ tanks that either contained (experimental "E") or did not contain (control "C") 100 µg/100 ml carrier T_3. After 6 days fish were immersed for 6 or 24 hr in plastic-lined glass aquaria that contained 25-1 of $*T_3$ with levels of carrier T_3 similar to those to which they had been exposed previously.

Fish weights as well as liver weights (Table 3) were similar with minimal variations between all 4 groups of fish. Total gall bladder radioactivity (cpm/g BW) was comparable between all groups of fish. Total liver nuclear $*T_3$ (cpm) was much lower in fish immersed for 24 hr than 4 hr. Similar levels of saturable nuclear T_3 and B/F ratios were observed at 4 and 24 hr. B/F ratios were unaffected by added carrier T_3 (C vs E) indicating that saturable T_3-binding sites had not been demonstrated by this method.

4. To demonstrate saturable liver nuclear T_3-binding, increased carrier T_3 should decrease nuclear $*T_3$. The previous experiment, based on B/F ratios, indicated that it was impractical to immerse fish in $*T_3$ to label liver nuclear T_3-binding sites. The objective was to establish a method, applicable to large numbers of T_3-immersed fish, that would demonstrate maximal displacement of nuclear

TABLE 3.   Influence of ambient carrier T_3 (100 µg/100 ml) on uptake of ambient *T_3 into liver nuclei (B/F) of trout[1] immersed for 4 or 24 hr. E = carrier treated; C = control, no carrier.

Parameter	4 hr		24 hr	
	C	E	C	E
Fish Wt. (g)	81.48 (2.95)	80.43 (2.00)	79.15 (3.45)	81.62 (2.14)
Liver Wt. (g)	1.04 (0.051)	0.91 (0.032)	0.95 (0.047)	0.86 (0.018)
Nuclear *T_3 (cpm)	815 (148)	893 (138)	119 (28)	113 (11)
B/F$_3$ (x 10^3)	18.09 (0.97)	15.66 (0.73)	13.54 (1.80)	13.93 (1.34)
Ambient radioactivity (cpm/ml)	1081 (20.9)	1119 (37.4)	935 (16.7)	927 (10.5)
Gall bladder *T_3 (cpm/g BW)	1659 (70)	1548[2] (183)	1641 (262)	1770[3] (60)

[1] $\bar{x}$, (SEM); n = 6.

[2] n = 4

[3] n = 5

*T_3 by carrier T_3 <u>in vivo</u> with a minimum of intragroup
variation.

Four different methods of administering stable T_3
and *T_3 were examined:

(i) fish were injected (Van der Kraak and Eales,
1980) with *T_3 (0.17 μCi, 5.9% *I^-) (control = C) or with
*T_3 (0.17 μCi, 5.1% *I^-) and 5000 ng carrier T_3 (experimental =
E).

(ii) fish were immersed in water with 100 μg/100 ml
carrier T_3 (E) or in water without carrier T_3 (C) for 7
days, then C-fish were injected with *T_3 (0.13 μCi, 3.28%
*I^-) and E-fish were injected with *T_3 (0.13 μCi, 3.07%
*I^-) and 5000 ng carrier T_3;

(iii) fish were immersed in water with 100 μg/100 ml
carrier T_3 (E) or in water without carrier T_3 (C) for 7
days, then C-fish were immersed in aquaria containing
25-1 of water with *T_3 (0.054 μCi/100 ml, 10.62 *I^-) and
E-fish were immersed in similar aquaria with *T_3 (0.054 μCi,
6.49% *I^-).

(iv) fish were immersed in water with 100 μg/100 ml
carrier T_3 (E) or without carrier T_3 (C) then all fish
were injected with *T_3 (0.25 μCi, 2.13% *I^-).

Prior to T_3 or *T_3 treatment all fish were acclimated
for 3 days in flowing water. Fish were bled and killed
12 hr after their initial exposure to *T_3 and nuclei were
obtained thereafter.

Fish weights, liver weights, and liver DNA content were comparable between all groups. In experiments (i) and (ii) (Fig. 5) N/L ratios of E-fish were approximately 1/3 those of C-fish demonstrating saturable liver nuclear T_3-binding sites. In experiment (iii) saturable binding was not observed so immersion of fish in $*T_3$ was again shown to be ineffectual in labelling liver nuclear T_3-binding sites. In experiment (iv) saturable liver nuclear T_3-binding was demonstrated with minimal intragroup variation. N/L ratios for experimental fish were similar between experiments and N/L ratios consistently reflected the proportion of liver nuclear radioactivity between C and E fish (Table 4). Gall bladder radioactivity was similar between C and E fish within each experiment, except (iii), implying equivalent exposure of fish to $*T_3$.

Routinely, radioactive samples of blood and sometimes nuclei were examined on G-25 sephadex separation columns to evaluate the proportion of radioactivity due to $*I^-$. In the case of fish immersed in carrier T_3 then injected with $*T_3$ (iv), $*I^-$ contamination of injectable material was 2.1% (n = 2; SEM = 0.002) while plasma from C-fish and E-fish contained 9.5 and 6.8% $*I^-$ (n = 8, SEM = 0.81 and 0.34) respectively. Nuclei of C-fish (n = 2, $\bar{x}$ = 4.42% $*I^-$, SEM = 3.22) and E-fish (n = 2, $\bar{x}$ = 4.15% $*I^-$, SEM = 0.76) fish had similar low $*I^-$ levels.

50

FIGURE 5. Examination of several protocols for the administration of *T_3 and carrier T_3 to rainbow trout (n=6). In all cases fish were bled and killed 12 hr after their initial exposure to *T_3. Then liver nuclei were isolated and counted for radioactivity. Nuclear radioactivity (N) was compared to total liver radioactivity (L). Each bar represents the mean ($\pm$2 SEM) of 6 fish).

i. Fish were injected simultaneously with *T_3 (Control = C) or with *T_3 and 5000 ng carrier T_3 (Experimental = E).

ii. Fish were immersed in water without carrier T_3 (C) or with 1 μg/ml carrier T_3 (E), then injected with *T_3 (C) or with *T_3 and 5000 ng carrier T_3 (E).

iii. Fish were immersed in water without carrier T_3 (C) or with 1 μg/ml carrier T_3 (E), then immersed in 25-1 aquaria with *T_3 (C) or with *T_3 and 1 μg/ml carrier T_3 (E).

iv. Fish were immersed in water without carrier T_3 (C) or with 1 μg/ml carrier T_3 (E) then injected with *T_3.
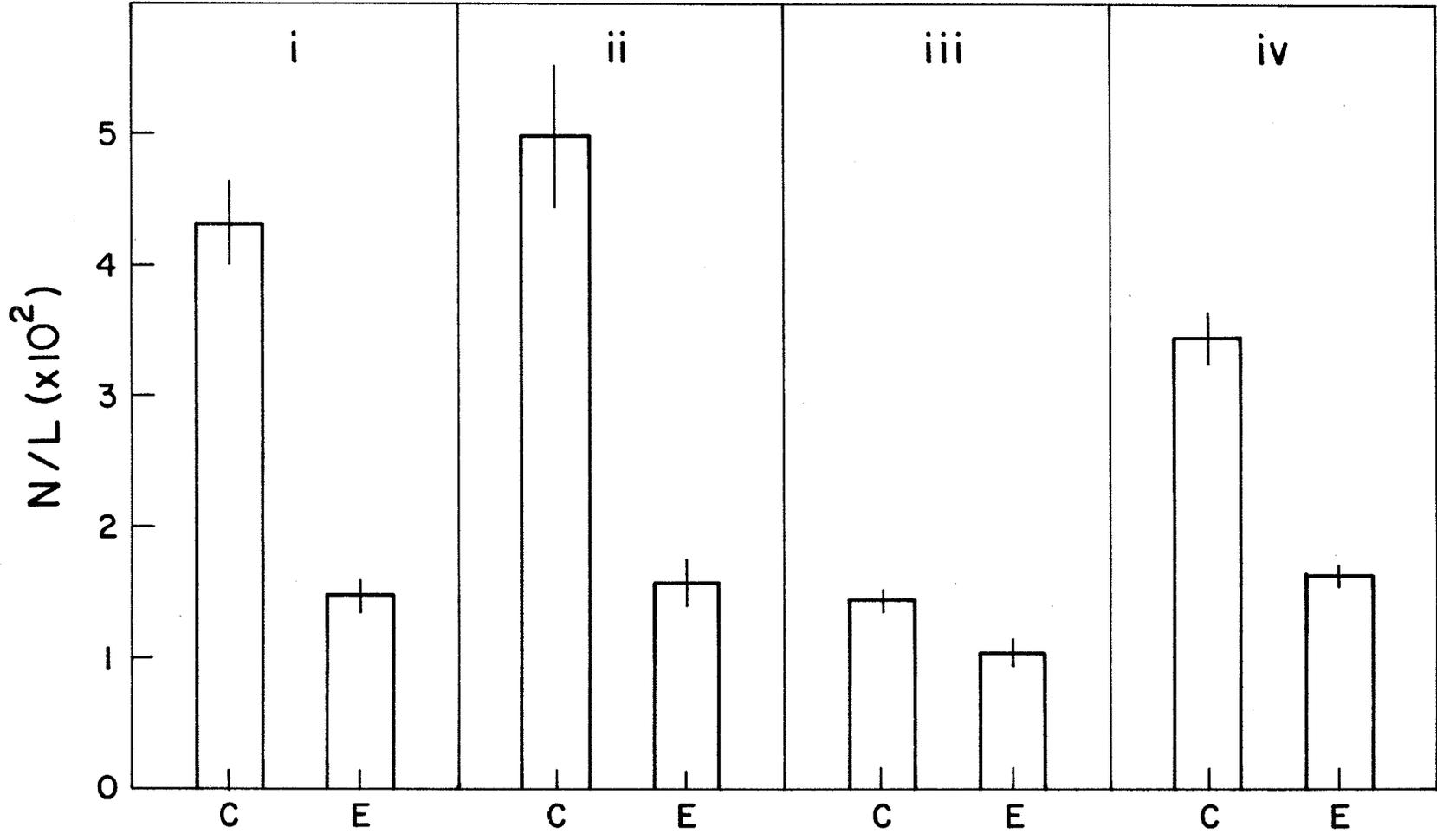
TABLE 4. Influence of different protocols for T_3 and $*T_3$ administration to trout on liver nuclear and gallbladder radioactivity (cpm). E = carrier treated; C = control, no carrier.

Expt.	Total liver nuclear radioactivity		Gallbladder radioactivity	
	C	E	C	E
i	671 (68)	260 (22)	28526^2 (5848)	39437 (4078)
ii	617 (55)	171 (33)	34928 (2882)	32700 (4277)
iii	64 (10)	35 (16)	57627 (6269)	26413^2 (2063)
iv	1046 (72)	454 (19)	50708 (3205)	42389^2 (5307)

[1] $\bar{x}$, (SEM); n = 6

[2] n = 5

Based on the above results (experiment iv) the following protocol was established for future experiments.

i. Fish were sorted then acclimated in 150-l fibreglass tanks and maintained on 0.5-1.0% BW rations daily for 3 days.

ii. Fish were immersed in a static solution of T_3, recharged daily for 7 days. Feeding was discontinued three days prior to injection of $*T_3$.

iii. Fish were netted, anaesthetized, bled, intraperitoneally injected with $*T_3$ and then returned to their respective tanks.

iv. Twelve hr after $*T_3$ injection fish were netted, anaesthetized, bled, killed and tissue samples and nuclei obtained.

5. Given that a satisfactory method for labelling liver nuclei in vivo was developed it was necessary to determine the time after $*T_3$ injection for maximum binding of $*T_3$ to saturable sites. Fish were sorted and maintained in flowing water for 3 days at a ration of approximately 0.5% BW. Thereafter fish were immersed as previously described for 7 days in a solution of 25 μg/100 ml carrier T_3 (T_3-fish), or without a nuclear saturating dose of carrier T_3 (C-fish). Three days prior to injection fish were starved. On the

beginning of the eighth day of immersion fish were injected

with $*T_3$ (0.33 µCi, 3.3% $*I$), returned to their tanks

and sampled for plasma and liver nuclei at 3, 6, 12 and 24

hr thereafter.

Fish weights, liver weights and liver DNA content

were comparable between all groups. Total liver nuclear

radioactivity (Fig. 6) for both control (C-fish) and

(T_3-fish) groups was maximal at 6 hr post-injection, and

declined thereafter. To determine saturably bound T_3,

nuclear radioactivity of T_3-fish was subtracted from that

of control fish (Fig. 6). Maximal saturable $*T_3$ binding

occurred at 12 hr post-injection.

B/F ratios (Fig. 6 II) for T_3-fish did not vary between

3 and 24 hr post-injection while ratios for C-fish increased

gradually. Subtraction of average T_3 B/F ratios from C

values yields the B/F ratio for saturable liver nuclear T_3

binding (Fig. 6 II). Saturably bound T_3 B/F ratios increased

regularly to 12 hr then more gradually to 24 hr post-injection.

Liver:plasma (L/P) ratios (Fig. 6 III) for both

C- and T_3-fish increased in parallel to 12 hr post-injection

with no change thereafter.

Since maximal binding of $*T_3$ to saturable liver

nuclear sites occurred 12 hr after $*T_3$ injection all fish

used in subsequent binding studies were sampled 12 hr

post-injection.

54

FIGURE 6. Trout were acclimated in running water for 3
days then immersed in static water that
contained (T_3) or did not contain (C) a nuclear
saturating dose of carrier T_3 (25 µg/100 ml).
Subsequently fish were intraperitoneally
injected with $*T_3$ and killed at preset times
thereafter. Nuclei, liver and the T_3 fraction
(HP-fraction) of fish plasma were counted
for radioactivity. Each point represents
the mean ($\pm$ 2 SEM) of 6 fish.

I. Total liver nuclear radioactivity,
    assumed to be $*T_3$. C-T_3 defines the
    time course of $*T_3$ uptake by liver nuclear
    saturable T_3-binding sites.

II. B/F ratios

III. L/P ratios ($\frac{\text{Total liver cpm}}{g} / \frac{\text{plasma cpm}}{g}$)
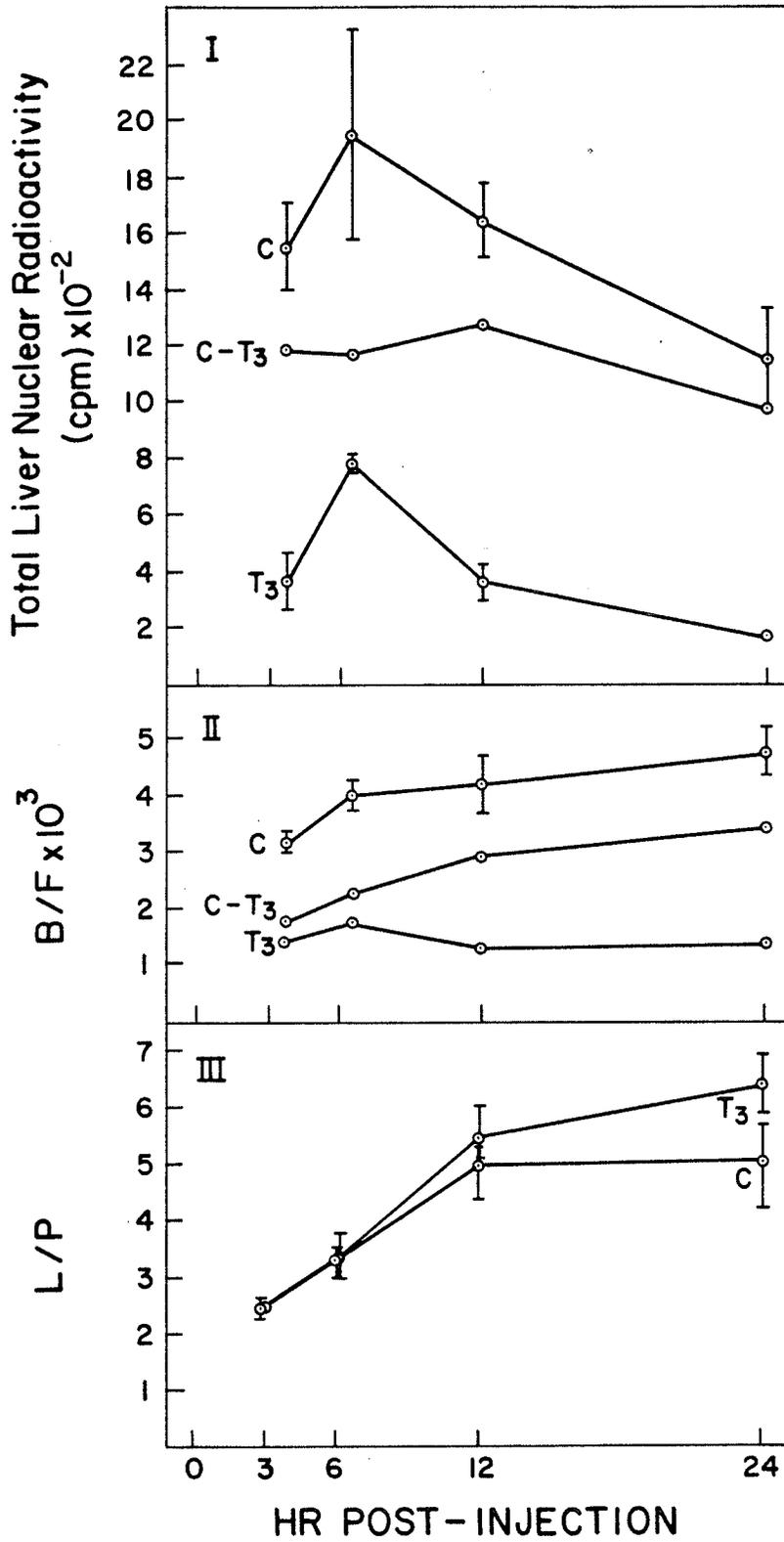
B. DETERMINATION OF CHARACTERISTICS OF NUCLEAR T_3-BINDING

   SITES IN LIVER

The purpose of this experiment was to chronically elevate fish plasma T_3 levels, thereby establishing basal, partially saturated and fully saturated liver nuclear saturable T_3-binding sites. To this end estimates of the influence of ambient and plasma T_3 concentration on K, MBC, and nuclear occupancy of saturable T_3-binding sites were determined.

Groups of fish were acclimated in standard fashion and then immersed in T_3 (0, 1.25, 2.5, 5.0, 10, 25, 50 or 100 µg/100 ml;Competition Study I). Fish were bled, injected with *T_3 (0.27 µCi, 4.63% *I^-), and after 12 hr bled, killed and nuclei obtained. In a parallel experiment, Competition Study II, fish were similarly treated, immersed in carrier T_3 (0, 0.125, 0.250, 0.750, 1.00, 2.00, 10, or 50 µg/100 ml T_3) and injected with *T_3 (1.60 µCi, 9.35% *I^-).

Fish weights ($\bar{x}$ = 101.92 g, SEM = 1.4), liver weights ($\bar{x}$ = 0.94 g, SEM = 0.02), and liver DNA content ($\bar{x}$ = 3.019 g DNA/kg liver, SEM = 0.395) for fish in Competition Study I were similar to fish weights ($\bar{x}$ = 100.6 g, SEM = 1.2), liver weights ($\bar{x}$ = 0.95, SEM = 0.02) and liver DNA content ($\bar{x}$ = 2.905 g DNA/kg liver, SEM = 0.068) for all 48 fish examined in Competition Study II.

Plasma T_3 levels increased as a function of ambient T_3 concentration (Fig. 7). The same data are replotted in

56

FIGURE 7.   Plasma T_3 concentrations of fish, in two
            experiments (Competition Studies I and II,
            immersed for 7 days in various concentrations
            of carrier T_3.   Each point represents a mean
            ($\pm$ 2 SEM) of five to ten fish.
            Body weights and plasma T_3 values are given
            in Appendix 5.
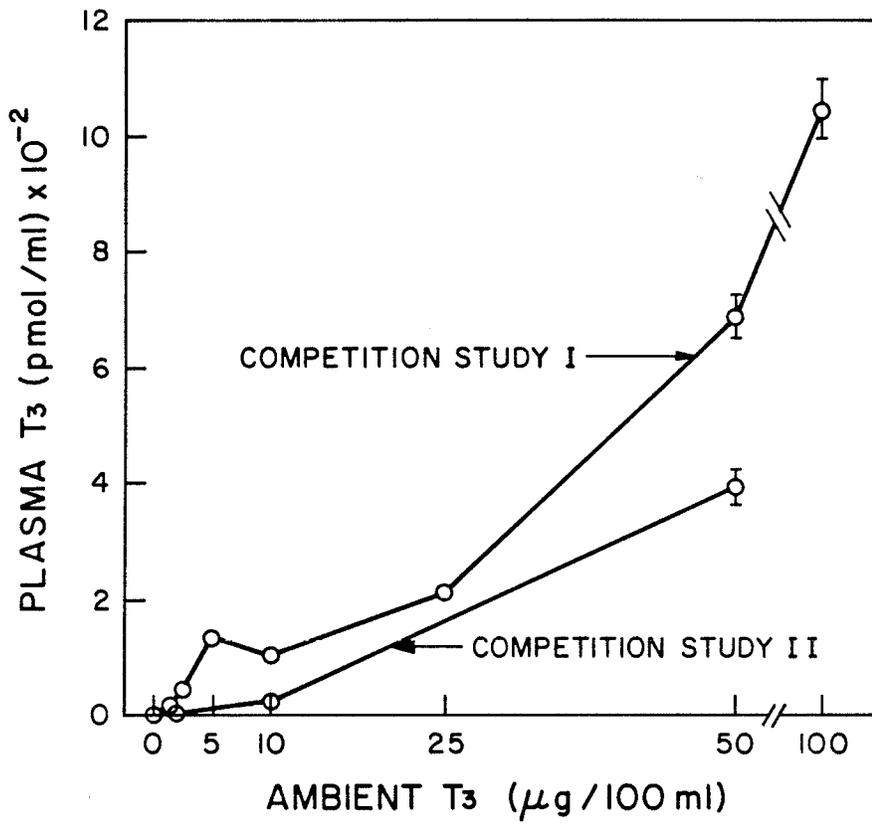
Fig. 8 as log plasma T_3 (pmol/ml) as a function of log ambient T_3 (pmol/ml). Assuming a linear relationship between these two parameters, linear regression generated best fit lines are:

log plasma T_3=0.898 log ambient T_3 + 0.184; r=965 for Competition Study I. and

log plasma T_3=1.162 log ambient T_3 - 0.902; r=0.949 for Competition Study II.

In the Scatchard plot (Fig. 9) B/F ratios of the first group of fish (Competition Study I) indicated that saturation of saturable T_3-binding sites occurred at the lowest imposed hormone concentration, 1.25 µg/100 ml.

Saturable (S) and Non-Saturable (NS) components of total *T_3-binding to liver nuclei for the second group of fish (Competition Study II) is demonstrated in the Scatchard plot (Fig. 10). Subtraction of the average B/F ratio of the non-saturable component from the saturable component of total *T_3-binding yields the corrected B/F ratios of saturable T_3-binding. The linear relationship between corrected B/F values and nuclear bound (B) T_3 (between 20 and 390 pmole T_3/kg liver) was described by the linear regression:

B/F = (-6.03 x 10^7 kg liver/mole T_3) x + 0.033; r = -0.74.

A Wilkinson plot (Fig. 11) was used to calculate K and MBC. F/B values, Free (F) liver T_3 divided by saturably bound (B) nuclear T_3, were plotted as a function of F and the linear relationship was described by the linear regression:

F/B = (2.57 x 10^9 kg liver/mole T_3) x + 43.7; r = 0.62.

The equilibrium constant and maximum binding capacity were:

K = 5.88 x 10^7 kg liver/mole T_3 = 1.71 x 10^7 g DNA/mole T_3, and

MBC = 389 pmole T_3/kg liver = 134 pmole T_3/g DNA.

58

FIGURE 8.   Logarithm of plasma T_3 level as a function

of the logarithm of ambient T_3 concentration.

Each point represents a mean ($\pm$ 2 SEM) of

five to ten fish.   Assuming a linear

relationship, linear regressions are

  log plasma T_3 = 0.898 log ambient T_3 (pmole/ml) + 0.184;

  r = 0.965
            for Competition Study I and

  log plasma T_3 = 1.16 log ambient T_3 (pmole/ml) = 0.841;

  r = 0.949
            for Competition Study II.

Body weights and plasma T_3 values are given in

Appendix 5.
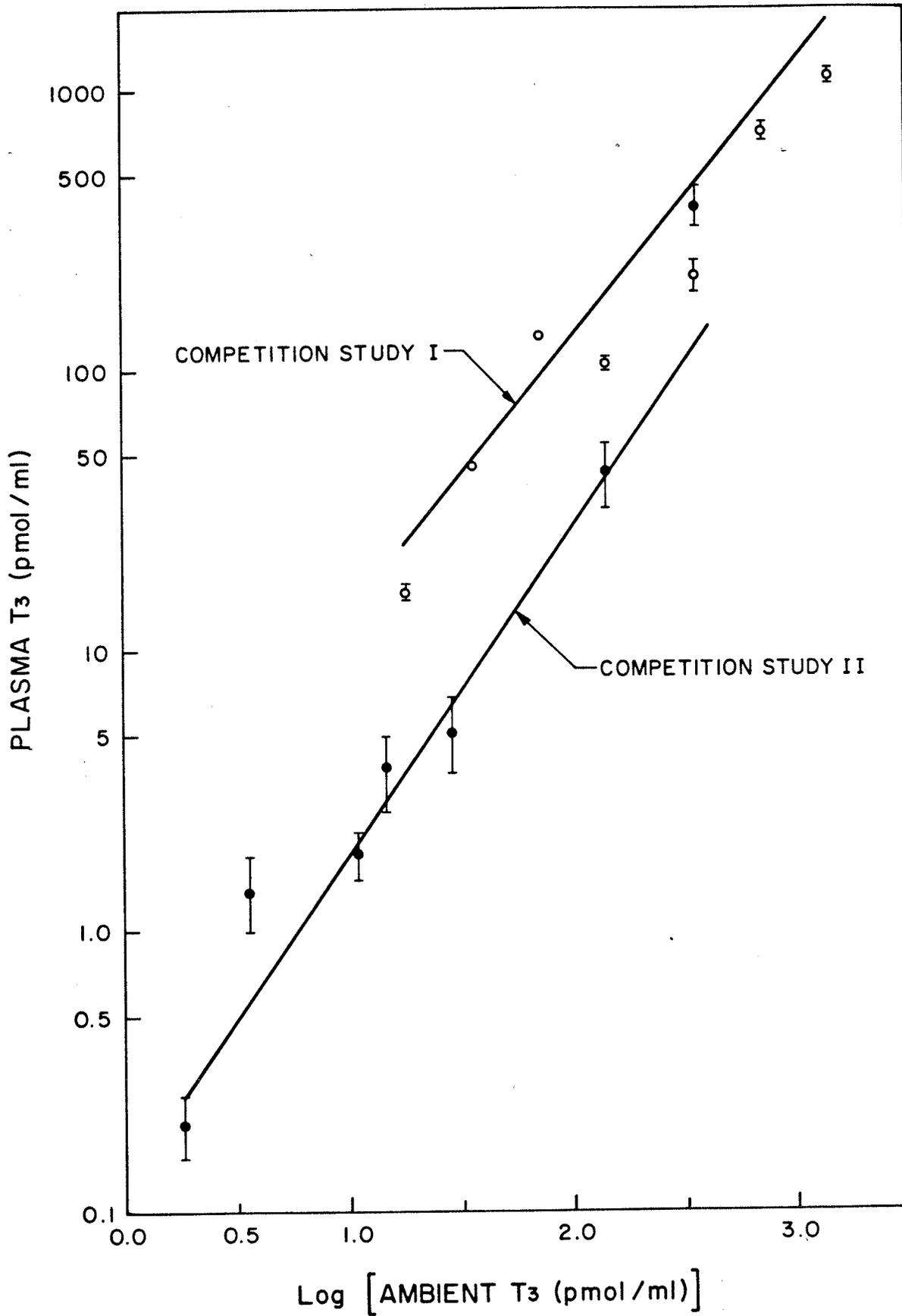
FIGURE 9. T_3-binding to trout liver nuclei (Competition

Study I). Scatchard plot demonstrating

saturable (S) and non-saturable (NS) binding

in which B/F is plotted against nuclear bound

T_3.

Each point represents the mean ($\pm$ 2 SEM) of

six fish.

NUCLEAR T3 (nmol T3 / kg liver)
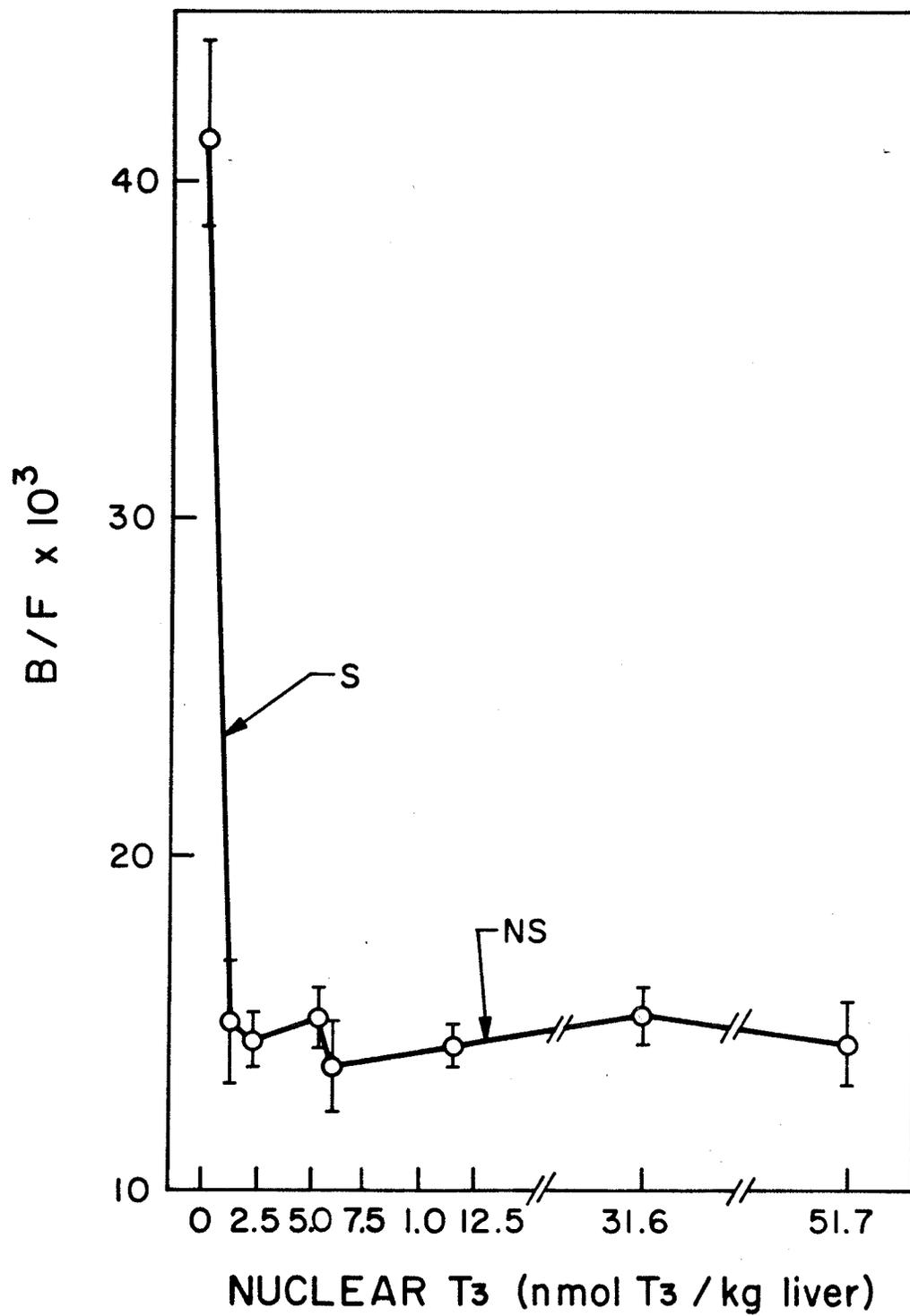
Figure 10. Scatchard plot of T_3-binding to trout liver nuclei (Competiton Study II). Saturable (S) and Non-Saturable (NS) T_3-binding sites are described by the plot of B/F as a function of nuclear bound T_3. Each point represents the mean ($\pm$ 2 SEM) of six fish. The linear regression for saturable binding is:

$$y = (-6.03 \times 10^7 \text{ kg liver/mole } T_3) \ x + 0.033; \ r = -0.740.$$

B/F and nuclear bound T_3 values are given in Appendix 6.
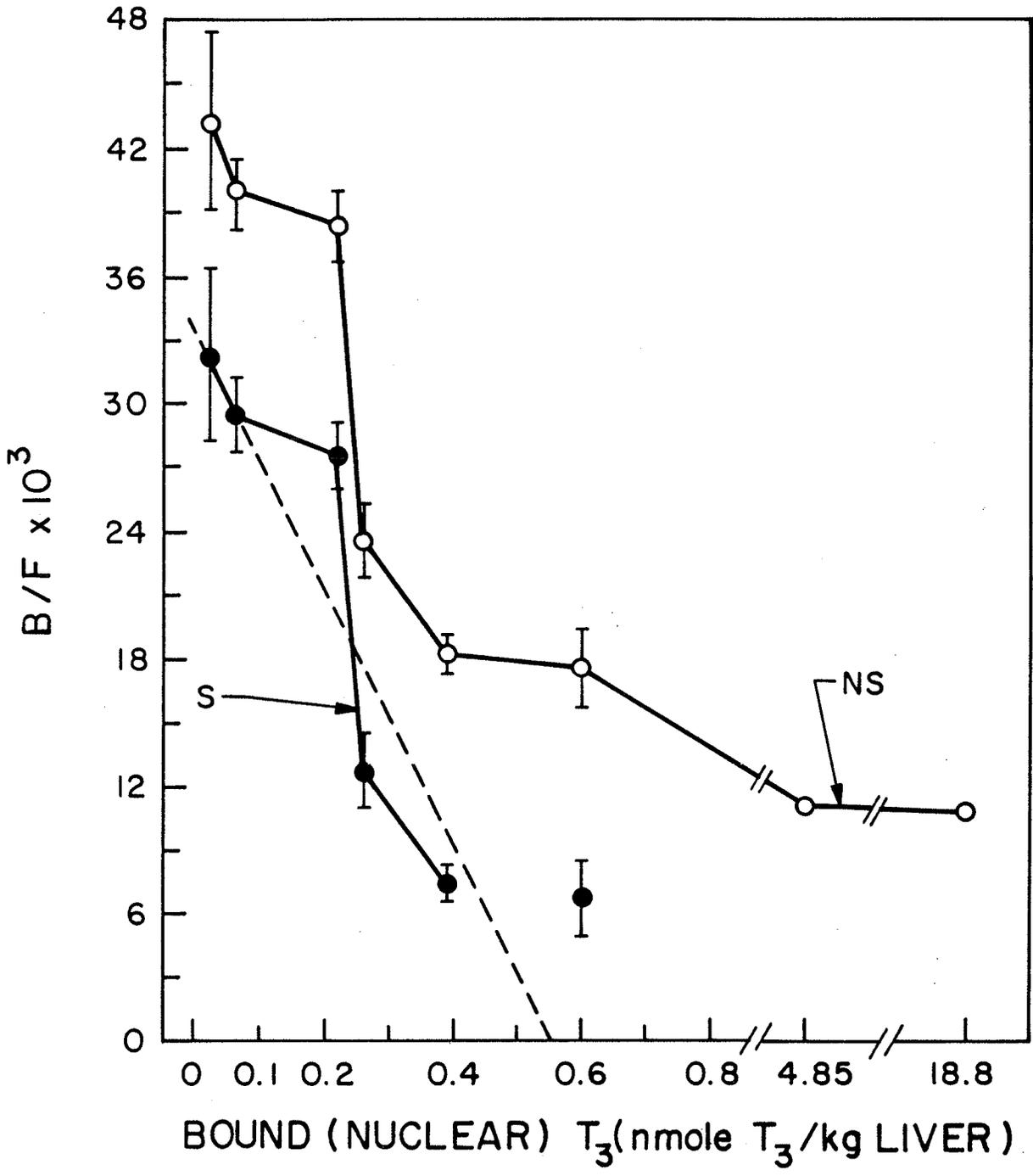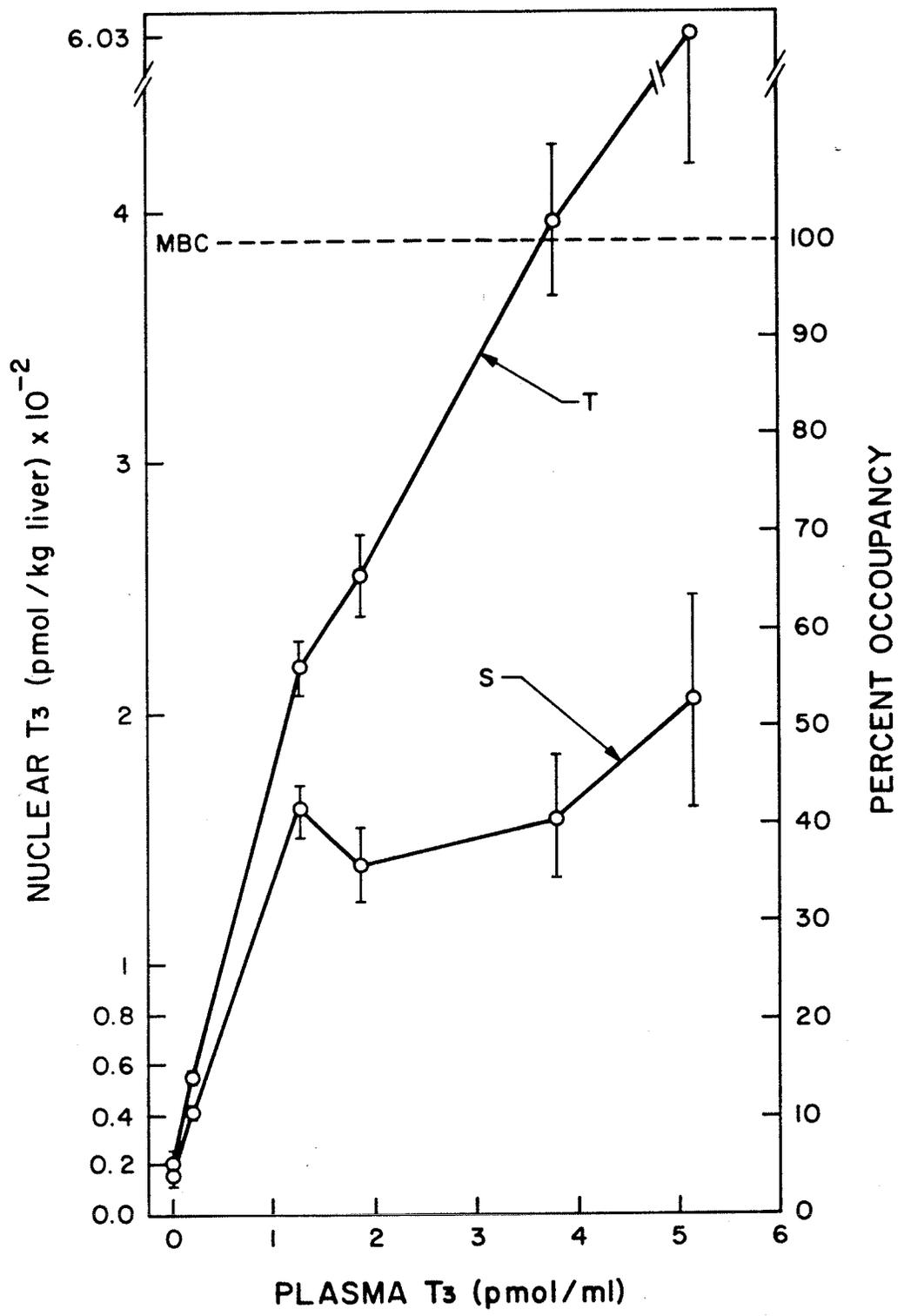
Figure 11. Wilkinson plot of T_3-binding to trout liver

nuclear saturable sites (Competition Study

II). The linear regression for saturable

binding is:

y = (2.57 x 10^9 kg liver/mole T_3) x + 43.7; r = 0.623.

The equilibrium constant (K) and maximum binding

capacity (MBC) are:

MBC = 389 pmole T_3/kg liver = 1.339 pmole T_3/g DNA

and

K = 5.88 x 10^7 kg liver/mole T_3 = 1.708 x 10^8 g DNA/mole T_3.

F/B and Free liver T_3 (F) values are given in

Appendix 6.
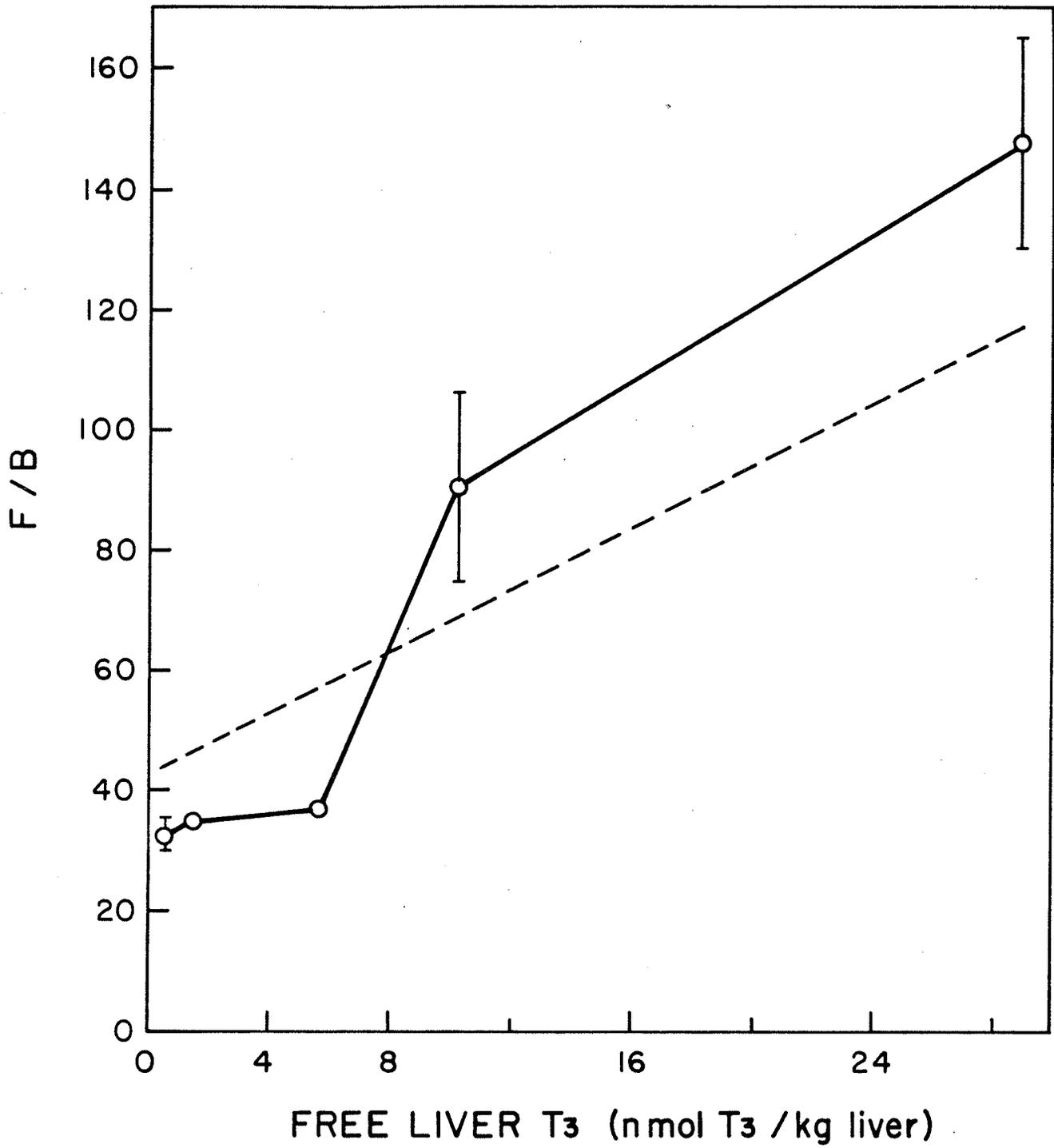
Total and saturably bound nuclear T_3 (pmole T_3/kg liver) were plotted as a function of plasma T_3 (pmole T_3/ml) (Fig. 12). Saturably bound nuclear T_3 constituted a progressively smaller proportion of totally bound nuclear T_3 with increasing plasma T_3 levels. All points in the curve of saturably bound T_3 occur at commonly observed plasma T_3 levels in rainbow trout. Fifty % occupancy occurs at approximately 5 pmole T_3/ml plasma. Saturation of nuclear sites is described at plasma T_3 levels as low as 16 pmole T_3/ml (Competition Study I), but no valid estimate of the plasma T_3 levels required to just induce saturation may be made.

C. RESPONSES OF FISH TO ELEVATED PLASMA T_3 CONCENTRATIONS

An attempt was made to elevate plasma T_3 levels such that saturable liver nuclear T_3 binding sites would be partially or totally saturated. The levels of gill and liver Na^+/K^+-ATPase and integumentary guanine were then measured in these fish and control fish with unaltered plasma T_3 levels. The attempt was to determine if there is a correspondence between occupancy of saturable liver nuclear T_3-binding sites and a physiologic response.

1. Fish with naturally low endogenous plasma T_3 concentrations were immersed, as previously described, in one of a solution of 0, 1.25, or 10 µg T_3/100 ml. After 7 days

FIGURE 12. Influence of plasma T_3 concentration on total (T) and saturable (S) T_3-binding to trout liver nuclei. Saturably bound T_3 (pmole/kg liver) expressed as a fraction of maximum binding capacity (MBC) represents occupancy of saturable sites. Each point represents the mean ($\pm$ 2 SEM) of six fish except when plasma $T_3 \simeq 5$ pmole/ml where n = 5 for saturably bound nuclear T_3. Values for nuclear bound T_3 and plasma T_3 concentrations are given in Appendix 6.
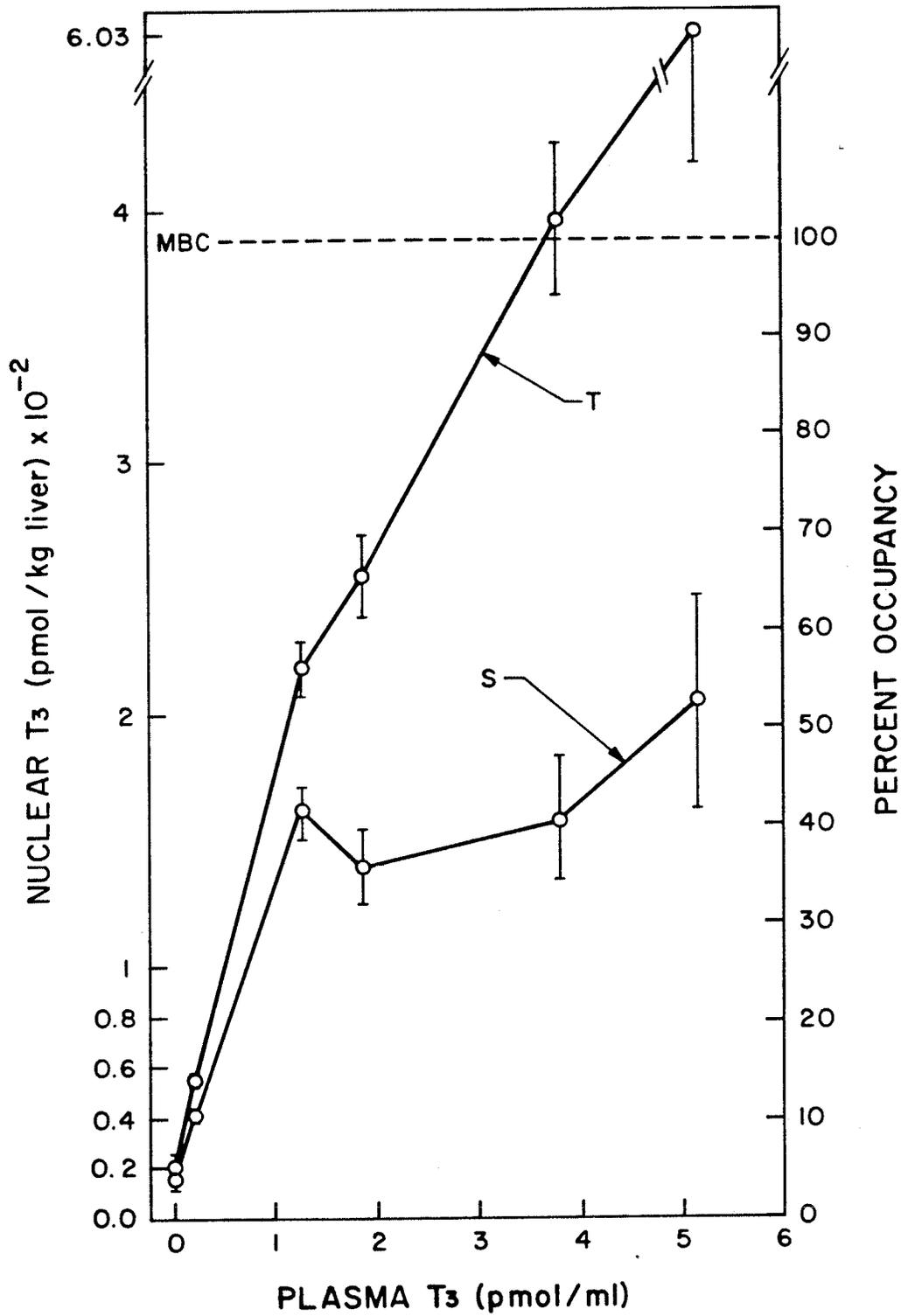
fish were bled and assayed for gill and liver Na^+/K^+-ATPase activity.

Plasma T_3 increased with ambient T_3 concentration (Fig. 13). Plasma T_3 levels of 0.5, 8.8 and 73 pmol T_3/ml are predicted to have induced binding site occupancies ranging from negligible levels (<5%), to between 50 and 100%, and ultimately to saturation. Liver Na^+/K^+-ATPase activity increased significantly (p, 0.01) (from 0.318 to 0.473 µmole Pi/hr/mg protein) with plasma T_3 concentration (Fig. 14). In contrast, at similar plasma T_3 levels, gill Na^+/K^+-ATPase activity (Fig. 14) decreased signficantly (p, 0.05) from 5.7 to between 4.5 and 4.6 µmole Pi/hr/mg protein.

2.   In a second experiment fish were immersed, in one of 0, 2 or 10 µg T_3/100 ml. After 6 days 10 fish were bled, killed and gill Na^+/K^+-ATPase activity determined. Remaining fish were immersed for a total of 20 days after which they were bled, killed and frozen in plastic bags for later extraction and determination of integumentary guanine.

Plasma T_3 increased with ambient T_3 concentration (Fig. 15). Plasma T_3 levels of fish exposed to similar hormone concentrations were comparable between 6 and 20 days immersion except for the 10 µg T_3/100 ml groups. Plasma T_3 concentrations (4.5, 9.7, 25 pmole T_3/ml) corresponded to

FIGURE 13. Plasma T_3 concentrations of fish immersed
in various concentrations of T_3 for 7 days.
Each point represents a mean ($\pm$ 2 SEM) of
10 fish. Plasma T_3 values are given in
Appendix 7.
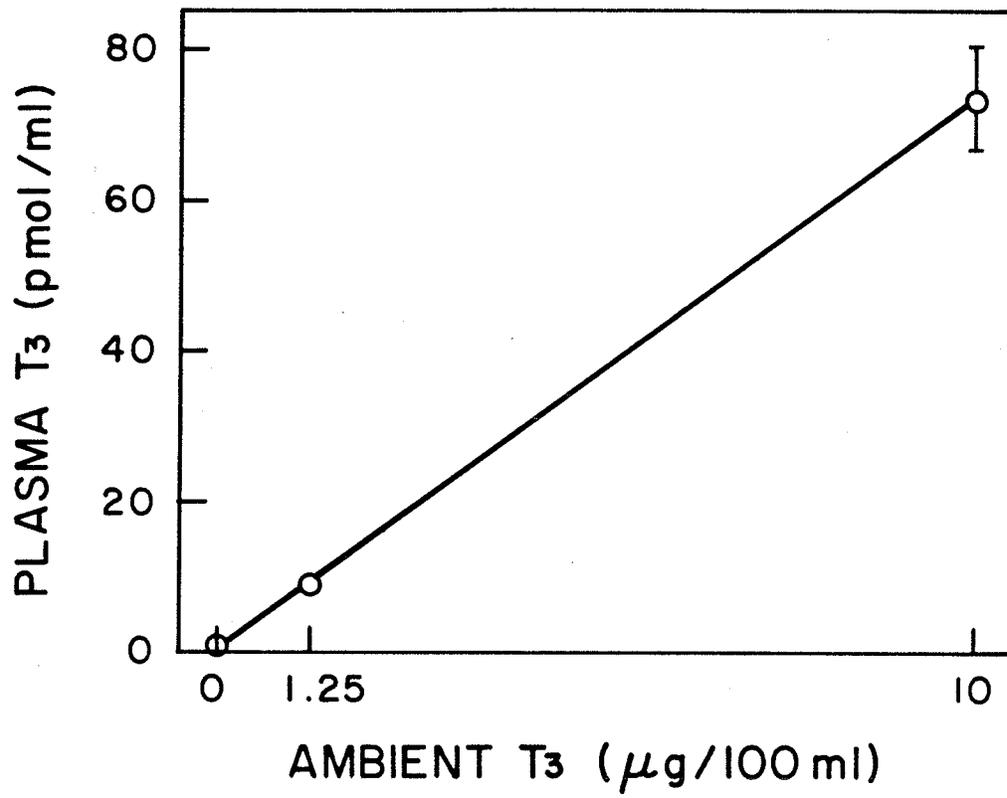
FIGURE 14.   Gill (O) and liver (▢) Na^+/K^+-ATPase
activity in fish with various plasma T_3
concentrations.   Each point represents
a mean ($\pm$ 2 SEM) of nine to ten fish.
Body weights and Na^+/K^+-ATPase activity
values are given in Appendix 7.
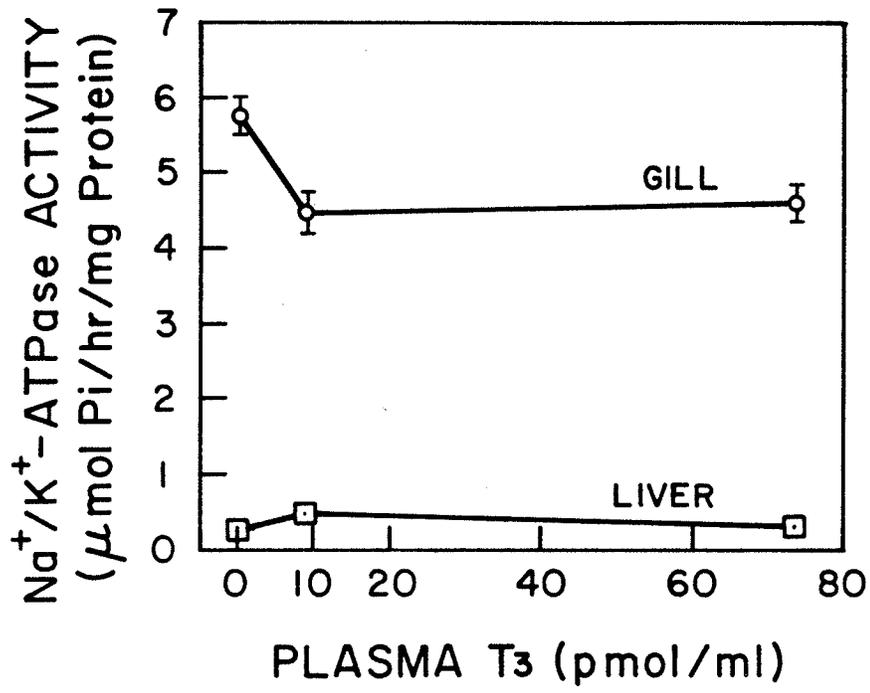
FIGURE 15.　Plasma T_3 concentrations of fish immersed for
6 (O) or 20 (□) days in various concentrations
of T_3.　Each point represents a mean ($\pm$ 2 SEM)
of nine to ten fish.

Body weights and plasma T_3 values are given in
Appendix 8.
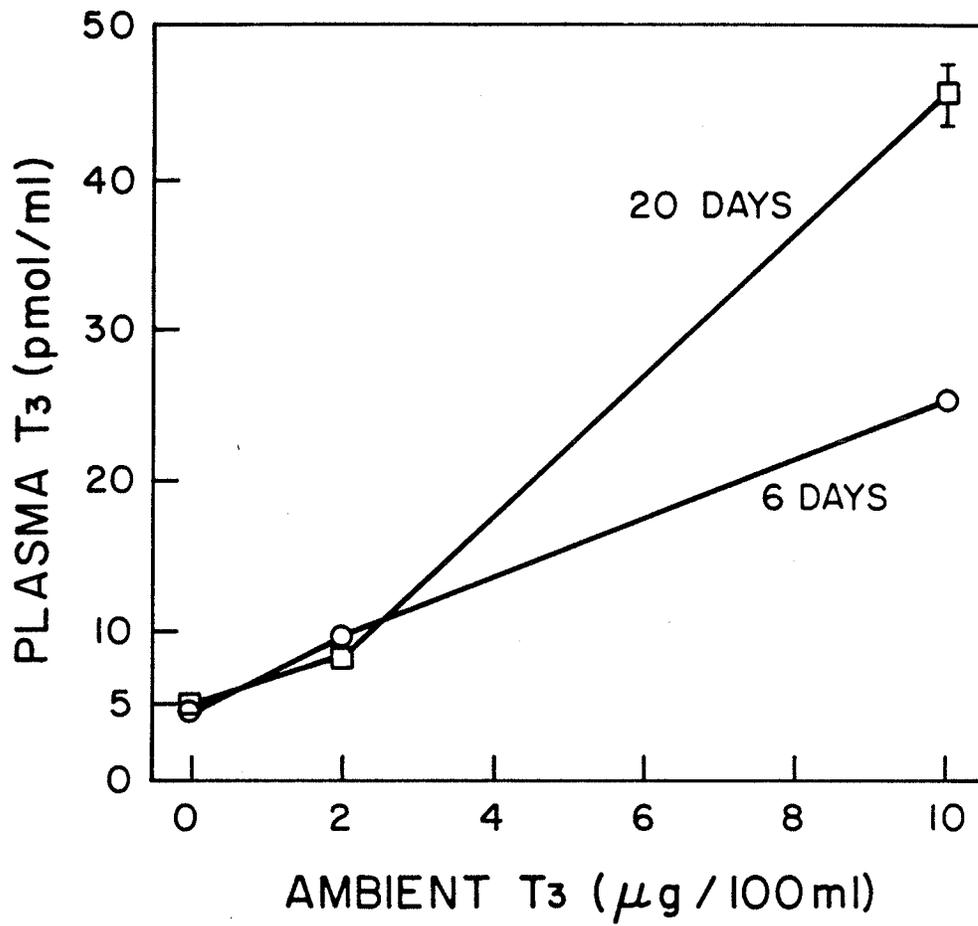
predicted occupancies of liver nuclear binding sites of
near 50%, between 50 and 100%, and well in excess of 100%.
Gill Na^+/K^+-ATPase activity (Fig. 16) decreased significantly
(p, 0.01) from 3.7 to 2.7 to 2.3 μmole Pi/hr/mg protein
with increasing plasma T_3 concentrations.

Integumentary guanine was not influenced significantly
(p, 0.05) by alterations in plasma T_3 levels (Fig. 16).

FIGURE 16. Gill Na^+/K^+-ATPase activity (O) and dermal

guanine concentrations (□) as a function of

plasma T_3 levels of fish immersed in T_3 for

6 (O) or 20 (□) days. Each point represents

a mean ($\pm$ 2 SEM) of 10 fish.

Body weights, and plasma T_3, gill Na^+/K^+-ATPase,

and dermal guanine values are given in Appendix

8.
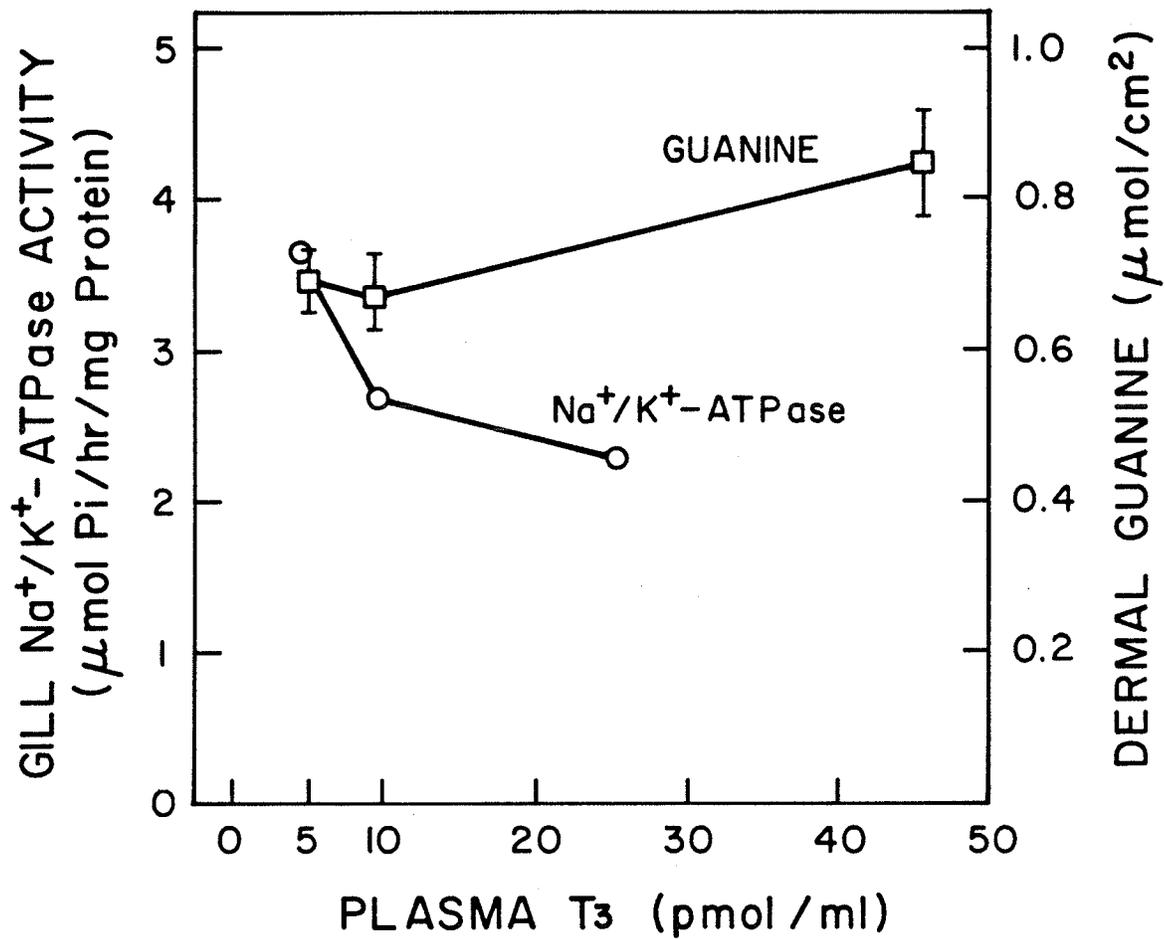
DISCUSSION

A. UPTAKE OF AMBIENT T_3

Immersion of trout in T_3 results in a rapid accumulation

of T_3 in plasma. This uptake phase (0-24 hr) is followed by

an adjustment phase in which plasma T_3 levels decrease

somewhat and then increase to a steady-state by 120 hr.

Plasma T_3 concentrations do not achieve the levels of

ambient T_3; generally steady-state plasma T_3 is one-tenth

the ambient T_3 concentration (Fig. 1). At relatively low

(0-10 μgT_3/100 ml) ambient concentrations, plasma T_3 levels

are a constant proportion of ambient T_3. Plasma T_3 is an

increasingly greater proportion of ambient T_3 at greater

ambient levels (Fig. 7).

Avenues for T_3 entry into immersed fish include:

gut surface due to water incidentally ingested with food,

surfaces of the eye, lining of the buccal cavity, integument

and gills. Both the integument and gills are in intimate

contact with the medium, highly vascularized and have

relatively large surface areas. However, on account of its

extremely large surface area and particularly large blood

flow it is likely that the gill represents the main site of

exchange. T_3 uptake across gill epithelia may be due

to active transport, passive diffusion, a combination of the

two or some other mechanism. These data do not allow for

speculation regarding a possible mechanism.

Plasma T_3 may be lost to a variety of tissues. In a given tissue, liver for example, T_3 may be free, or, bound to saturable and non-saturable sites (Fig. 3). Free T_3 that is lost from tissues in general may be returned to the plasma via interstitial fluid or elimated from the fish by degradation or excretion. Glucuronide-conjugated $*T_3$ lost from the liver to the gall bladder (Fig. 4) is secreted to the gut where it may be excreted with feces or absorbed into the plasma and recycled to the liver. It is plausible that T_3 in gill epithelial cells may be returned to the plasma or to the environment with the possibility of reabsorption. Muscle, a tissue accounting for the bulk of body weight, and the blood proteins, represent potentially enormous repositories for T_3.

Steady state plasma T_3 concentrations occur when factors contributing to plasma T_3 influx and efflux are equal. T_3 in blood may be free or bound to cellular and plasma proteins. By sequestration, blood proteins may significantly influence the concentration of T_3 in plasma.

Gill respiratory responses to physical activity of fish acclimating in T_3 solutions may potentiate a rapid initial accumulation of T_3 in plasma. Fish modify effective gill respiratory surface area by differential opening of operculae and alteration of blood flow to lamellae by selective constriction of blood vessels. Gill irrigation

is minimized to prevent osmotic stress associated with excessive ion loss and water uptake. During the first 24 hr of immersion fish exhibited considerable activity accompanied by increased respiratory demands and gill irrigation by T_3. Once acclimated to its new surroundings fish activity subsided, respiratory demands and T_3 irrigation of gills and T_3 uptake into plasma may have decreased (Fig. 1).

In contrast to plasma T_3, plasma $*T_3$ concentrations decreased markedly from maximal levels achieved after 4 hr immersion (Fig. 2) to a steady state at 95 hr. This apparent anomoly requires explanation. In the trout a large and slowly exchanged tissue pool of endogenous T_3 may dilute plasma $*T_3$ and depress plasma $*T_3$ after maximum levels are achieved. This explanation is supported by temporal differences in maximum $*T_3$ binding among tissues of differing potential for $*T_3$ sequestration. Maximum plasma, liver, and gall bladder radioactivity occur at 4, 8, and 24 hr respectively (Figs. 2, 3, 4).

In immersed fish, levels of ambient T_3 influence plasma T_3 concentrations. The relationship between plasma and ambient T_3 at low (0-2 µg/100 ml) ambient T_3 concentrations is linear. It is probable that the factors affecting removal of T_3 from plasma have greater potential influence over plasma T_3 concentrations than factors affecting influx. Factors such as ambient temperature may influence plasma thyroid hormone levels by modifing the rate of metabolism of thyroid

hormones (Eales, 1979b). For similarly-treated fish there
is a predictable and reproducible relationship between the
logarithm of steady state plasma T_3 concentration and the
logarithm of ambient T_3 concentration (Fig. 8). The
relationship is applicable to estimation of ambient
concentrations required to produce desired plasma T_3 levels.
With increased ambient T_3 concentrations the factors governing
T_3 influx into plasma increase in their potential to modify
plasma hormone concentrations. Variation in plasma T_3
decreases within a group and between groups with increased
ambient T_3 (Fig. 8). This may be the result of the decreasing
proportional influence of endogenous T_3 on plasma T_3 levels.
The linear regressions of the logarithm of plasma T_3 as a
function of a logarithm of ambient T_3 converge with
ambient T_3 concentration. These results may imply that
certain fundamental factors controlling T_3 influx into plasma
become progressively more dominant with increased ambient T_3.
The predominant factor affecting T_3 input into plasma of
immersed fish, likely, may be ambient T_3 concentration.

There are advantages and disadvantages associated with
immersing fish in T_3 as a protocol for the chronic elevation
of plasma T_3. Immersion allows for uniform treatment of
large numbers of fish. Stress is minimized due to minor
handling of fish during treatment. Sensitive control over
ambient dosage is possible. Fish may be exposed to a constant

dosage for prolonged ($\leq$ 20 days) periods. Ultimately,
desired plasma T_3 concentrations, within tolerable limits,
may be reproducibly established.

In order to successfully immerse trout in static T_3
solutions overcrowding must be avoided and water quality
preserved. Stress associated with crowding and resulting
poor water quality may lead to infections. When trout are
fed more than 0.5-1.0% of body weight, uneaten food and
feces may accumulate and jeopardize water quality. These
conditions necessitate frequent water changes with the
addition of considerable amounts of hormone. Fish are
immersed in static water so a controlled environment room
is required to maintain temperature. An important restriction
to this protocol is that it is applicable only to aquatic
organisms. This procedure does not lend itself to short-term
(less than 5 days) experiments because steady-state plasma
T_3 levels are achieved after 5-7 days. Immersion is more
appropriate for the chronic elevation of plasma T_3 levels.

To this author's knowledge no other information is
available on factors affecting the relationship between
ambient and plasma T_3 in fish. In order to successfully
apply the relationships described here it would follow that
conditions be duplicated as closely as possible. Conditions
may include: temperature, photoperiod, water quality as well
as ration and possibly other factors. Rainbow trout of a

similar genetic strain, size, age and sexual development,
(as sexual state influences plasma T_3, Hunt and Eales, 1979),
should be used.

Eales (1974) established chronically elevated plasma
T_4 levels in brook trout (S. fontinalis), black bullhead
(Ictalurus melas), and channel catfish (I. punctatus) by
immersion. Increased ambient temperatures resulted in
decreased plasma T_4 levels in spite of similar ambient T_4
concentrations. Feeding, species and body size also
influenced plasma T_4 levels. Immersion of brook trout in
radioactive T_4 resulted in steady state plasma T_4 levels
(Eales, 1974). Plasma T_4 was less than ambient T_4 due to
biliary excretion.

Etkin (1935, 1968) and Kaltenbach (1982) immersed
frog tadpoles in solutions of T_4 and described plasma:
immersion time profiles similar to those for T_3 described
here.

B.   ESTIMATION OF NUCLEAR OCCUPANCY BY T_3 DURING CHRONIC
     IMMERSION

A protocol for demonstrating saturable T_3-binding
in fish with artificially elevated plasma T_3 was developed.
Van der Kraak and Eales (1980) simultaneously injected $*T_3$
and a nuclear-saturating dose of T_3 to demonstrate saturable

T_3 binding (Fig. 5 (i)). However, that method was not
compatible with current objectives involving immersion of
fish. Injection of a nuclear-saturating dose of T_3 into
T_3-immersed fish would drastically alter the predicted
plasma T_3 levels. It has been shown that immersion of
fish in $*T_3$ (Fig. 5 (iii)) is an unsatisfactory method for
demonstrating saturable binding. However, injection of $*T_3$
into fish that were or were not immersed in T_3 (Fig. 5 (iv))
demonstrates saturable binding and is compatible with prolonged
immersion. Administration of $*T_3$ to fish by injection, rather
than by immersion, is a more efficient method for demonstrating
saturable $*T_3$-binding. This may be a consequence of ambient
T_3, more than injected $*T_3$, being subjected to multicompartment
sequestration. Despite the route of $*T_3$ administration,
demonstration of saturable sites (Fig. 5 E-values) is
accomplished by immersion in, or injection of sufficient T_3.

The optimal time for examining saturable $*T_3$ binding
is when it is maximal at 12 hr post-injection (Fig. 6).
This finding is similar to values reported by Van der Kraak
and Eales (1980). The high affinity of T_3 for saturable
sites is reflected by their B/F ratios which increase even
after maximum total and non-saturable $*T_3$-binding at 6 hr

post-injection. The cause may be the retention of $*T_3$
by saturable sites as opposed to general hepatic $*T_3$
removal. L/P ratios describe $*T_3$ flux between the liver
and plasma. Isotopic equilibrium between liver and plasma
exists at the time of sampling, 12 hr after T_3 injection,
as indicated by constant L/P ratios.

The time course of maximum nuclear $*T_3$ binding varies
among species. $*T_3$ uptake into liver nuclei of pre-metamorphic
tadpoles is greatest 2-7 hr after $*T_3$ injection (Toth and
Tabachnick, 1979) and is temperature dependent (Toth and
Tabachnick, 1980). In rats, maximum binding occurs between
0.5 hr (Oppenheimer et al., 1974a) and 2 hr (DeGroot and
Strausser, 1974) after $*T_3$ injection. Eales et al. (1983)
indicate that T_3 metabolic clearance rates are temperature
dependent. It may be this phenomenon which provides the
more gradual uptake at the low temperatures (9-12°C) at
which these fish were kept. Temperature may conceivably
affect the association of T_3 with saturable sites. Variations
in plasma and cytosolic T_3-binding proteins between these
classes of animals also may influence the time course of uptake.

Saturable $*T_3$ nuclear binding was demonstrated by
Scatchard analysis over a range of plasma T_3 concentrations
(Fig. 9, 10). For a monotypic population of saturable
binding sites Scatchard analysis theoretically produces a
distinct angular intersection between saturable and non-saturable

binding components (Scatchard, 1949). The relationship between B/F and B is curvilinear where there is more than one type of saturable site. In this study, the linear components of saturable and non-saturable binding (Fig. 10) have no discrete intersection but join curvilinearly. It is possible that chronic elevation of plasma T_3 modifies the displacement of *T_3 perhaps by increasing the number of T_3-binding sites (Nakamura et al., 1979). In the range of saturable binding, elevated plasma T_3 levels ranged from commonly observed to undetectable levels. Alteration of saturable binding may be detectable only in this range of chronically elevated plasma T_3. Presently, the consensus is that T_3 does not alter K or MBC in mammals.

Wilkinson analysis (Fig. 11) was used to calculate the equilibrium constant (K = 5.9 x 10^7 kg liver/mole T_3 = 1.7 x 10^8 g DNA/mole T_3) and maximum binding capacity (MBC = 389 pmole T_3/kg liver = 134 pmole T_3/g DNA). The methods for calculation of K in Scatchard and Wilkinson analyses are algebraically interchangable (Rodbard, 1973) but each method has different implied assumptions. Wilkinson analysis assumes uniformity of variance for F/B while the Scatchard plot assumes uniformity of variance for B/F. A portion of *T_3-binding to liver (F in the Wilkinson plot) that is non-specific makes a smaller proportional contribution to error than non-specific *T_3 binding to nuclei (B in the Scatchard plot).

Previous estimates of K (9 - 12 x 10^7 kg liver/mole T_3) and MBC (430 - 620 pmole T_3/kg liver) (Van der Kraak and Eales, 1980) are comparable to those described here. Variation in estimates may be a consequence of differences in age and nutritional status. Older fish and fed fish have MBC's greater than those of young (1 yr) or starved fish (Van der Kraak and Eales, 1980). Other influential factors may include prolonged exposure of fish to T_3 or staggering of T_3 and *T_3 administration (Rodbard, 1973).

The association constant (K_a = 1.03 x 10^9 M^{-1}) and MBC (127 pmole T_3/g DNA) were calculated from in vitro techniques for coho salmon liver nuclei (Darling et al., 1982). Equilibrium constants derived by in vivo protocols are influenced by the availability of free T_3 which depends on dissociation of T_3 from plasma and cellular proteins. Association constants are comparatively larger than equilibrium constants because of greater availability of free T_3 in in vitro incubation media.

Investigation of saturable T_3 binding in mammals largely has been conducted in vitro with rat tissues. Association constants may vary between 6.4 - 470 x 10^9 M^{-1} (Samuels et al., 1974a, and Oppenheimer et al., 1974a respectively) and may increase with age (Coulombe et al., 1979). MBC's are variable: 800 μg T_3/g liver (DeGroot and Torresani, 1975), 2.4 ng T_3/g liver (DeGroot and Strausser, 1974), 0.1 pmole T_3/mg DNA

(Surks and Oppenheimer, 1977) and are influenced by various physiologic states. These values and those reported for other classes of vertebrates (Table 1) are comparable to those reported here.

In this study 50% occupancy occurs at approximately 5 pmole T_3/ml plasma described as an endogenous level (4.2 pmole/ml) (Van der Kraak and Eales, 1980). Van der Kraak and Eales (1980) calculated saturation to occur at 22.5 pmole T_3/ml plasma compared to a concentration between 5 and 16 pmole/ml reported here. T_3 occupancy of a saturable site population is a function of the amount of saturably bound T_3 (Fig. 12) and depends on the amount of free T_3 available for binding. All tissues have access to plasma T_3 amd it is likely that saturable sites are monotypic. Consequently in a given fish the relationship between plasma T_3 content and % occupancy for one tissue is likely to be applicable to other tissues.

C. PHYSIOLOGIC RESPONSES TO T_3 TREATMENT

Ambient T_3 concentrations were selected to elevate plasma T_3 levels to coincide with nuclear T_3 occupancies varying from negligible to partially and totally saturated. Observed plasma T_3 levels did not correspond precisely to desired levels but did fall into acceptable ranges (Figs. 13, 15). It was assumed that saturable sites similar to those in liver

existed in cells of gill and integument. Occupancy rates
as a function of plasma T_3 levels were expected to be
similar.

Liver and gill Na^+/K^+-ATPase and integumentary guanine
content were examined as potentially T_3-responsive physiologic
parameters. Substantial evidence exists for the positive
influence of T_3 on mammalian liver Na^+/K^+-ATPase (Smith and
Edelman, 1979). Gill Na^+/K^+-ATPase is important to fish
osmoregulation (Skou, 1965; Kirschner, 1969) and there are
contradictory reports of T_3 influence (Dickhoff et al., 1982).
Integumentary guanine content, classically, has been associated
with thyroid activity (Premdas and Eales, 1976a).

Elevated plasma T_3 levels were accompanied by
significantly increased liver Na^+/K^+-ATPase activity (Fig. 14).
Liver binding site occupancy presumably ranged from negligible,
to 50-100%, to thoroughly saturated levels. In mammals T_3
has been shown to increase liver Na^+/K^+-ATPase activity both
in vivo and in vitro (Ismail-Beigi and Edelman, 1974a). The
levels of liver enzyme activity that are described here are
lower than those of mammals (Haber and Loeb, 1982). Reference
to the magnitude of fish liver Na^+/K^+-ATPase is unavailable.
As a result, it is unknown if the levels of activity reported
here are physiologically representative. Consequently, very
cautious interpretation of the influence of T_3 on fish liver
Na^+/K^+-ATPase is required.

Contrary to the trend in liver, gill Na^+/K^+-ATPase significantly and reproducibly decreased with increased plasma T_3 (Figs. 14, 16). Dickhoff et al. (1977, 1982) described a negative relationship between plasma T_4 and gill enzyme activity in T_4-immersed coho salmon (Oncorhynchus kisutch).

Many studies have examined the relationship between plasma thyroid hormone (T_4 or T_3) levels and gill Na^+/K^+-ATPase activity in salmonids (Zaugg and McLain, 1972; Zaugg and Wagner, 1973; Folmar and Dickoff, 1978, 1979; Dickoff et al., 1978b; Clarke et al., 1978; Giles and Vanstone, 1976; Saunders and Henderson, 1978) and an elasmobranch (Ginglymostoma cirratum) (Honn and Chavin, 1977). Positive correlations between plasma hormone levels and gill enzyme activities were the result of manipulations of temperature, photoperiod or salinity or the examination of captured smolts. It is conceivable that these factors directly and independently modified gill Na^+/K^+-ATPase activity.

Gill Na^+/K^+-ATPase activities reported here are comparable to those of chinook salmon (O. tshawytsha) (Ewing et al., 1979; Johnson et al., 1977), Atlantic salmon (S. salar) (McCartney, 1976), rainbow trout (McCarty and Houston, 1977), and various non-salmonids (Kirschner, 1969; Kamiya and Utida, 1968; Jampol and Epstein, 1970). Coho salmon have greater activities (Zaugg and McLain, 1970, 1971, 1976).

Trout liver and gill Na^+/K^+-ATPase have opposite responses to T_3-treatment. The mechanism by which T_3 effects changes in Na^+/K^+-ATPase may be tissue specific. Na^+/K^+-ATPase in gill is involved in ion transport and osmoregulation whereas in liver the enzyme has been associated with amino acid transport. Alternately, the concentrations of T_3 that the tissues are exposed to may be more influential in determining a response. Gill is exposed to ambient T_3 levels which are higher than the plasma levels to which the liver was exposed.

There appear to be phylogenic differences in the response of Na^+/K^+-ATPase to T_3. In mammals thyroid hormones increase Na^+/K^+-ATPase activity (Garg et al., 1982), whereas in amphibia (Bufo marinus; bladder) thyroid hormones do not influence Na^+/K^+-ATPase (Geering et al., 1982).

There was a pronounced trend of elevated guanine content with increased plasma T_3. However, there was no significant change in integumentary guanine content between fish with different chronically elevated plasma T_3 concentrations (Fig. 16). With sufficiently longer exposure a significant relationship may have emerged. It should be remembered that this is the first attempt to relate integumentary guanine content to chronically elevated plasma T_3.

Increased fish silveriness, associated with thyroid activity (Eales, 1979a), is a consequence of elevated

integumentary guanine content (Premdas and Eales, 1976a).
Repeated injection of bovine TSH into rainbow trout increases
liver production of guanine which is presumably incorporated
into the integument (Premdas and Eales, 1976b). Injected T_4
has no affect on guanine content but ambient thiourea, a
thyroid inhibitor, depresses integumentary guanine in brook
trout (S. fontinalis) (Chua and Eales, 1971). Temperature
more than photoperiod influence guanine content (Johnston and
Eales, 1967). Integumentary guanine levels reported here are
comparable to those previously described (Premdas and Eales,
1976b).

GENERAL CONCLUSIONS

1.   A predictable relationship between ambient and steady-state plasma T_3 concentrations was established for fish immersed in T_3 for 5-7 days.

2.   A method has been established for measuring K, MBC and occupancy of liver nuclear saturable T_3-binding sites in T_3-immersed fish; estimates of K and MBC were comparable to those previously described (Van der Kraak and Eales, 1980).

3.   Increased occupancy of saturable sites was significantly related to increased liver Na^+/K^+-ATPase and decreased gill Na^+/K^+-ATPase but could not be significantly related to integumentary guanine content.

4.   Several questions remain to be answered:

   (a) Do saturable T_3 binding sites exist in nuclei in other tissues of fish?

   (b) Does chronic elevation of plasma T_3 influence the rates of synthesis and degradation of saturable binding sites?

   (c) What are the consequences of chronic elevation of plasma T_3 in fish?

   (d) Is it the occupancy of an existing population of saturable sites or is it the absolute number of occupied sites that determines the occurrence or degree of a particular physiologic response?

SUMMARY OF SPECIFIC RESULTS

1. A protocol was established for chronically raising plasma T_3 levels in trout immersed in T_3 for 7 days.

2. The relationship between ambient and plasma T_3 was described by:

$$\log \text{plasma } T_3 = m \ (\log \text{ambient } T_3) + b.$$

3. Maximum $*T_3$ binding to saturable liver nuclear sites occurred 12 hr after $*T_3$ injection into fish held at approximately $11^{\circ}C$.

4. When $*I^-$ levels are between 6 and 9% of total plasma radioactivity over 96% of nuclear radioactivity is iodothyronine associated, presumably $*T_3$.

5. Saturable T_3-binding sites in liver nuclei
    $K = 5.88 \times 10^7$ kg liver/mole T_3 (1.71×10^8 g DNA/mole T_3), and MBC = 389 pmole T_3/kg liver (134 pmole T_3/g DNA).

6. Artificially elevated plasma T_3 levels, that are commonly observed, were related to occupancy of saturable liver nuclear sites up to 50%.

7. Plasma T_3 increased from 4.5 to 9.7 to 25 pmole T_3/ml, accompanied by significantly reduced gill Na^+/K^+-ATPase activity (3.67 to between 2.69 and 2.28 μmole Pi/hr/mg protein)(units).

Similarly, when plasma T_3 increased from 0.47 to 8.8 to 73 pmole/ml, gill Na^+/K^+-ATPase significantly decreased from 5.7 to between 4.5 and 4.6 units.

8. Liver Na^+/K^+-ATPase significantly increased from 0.318 to 0.473 units with plasma T_3.

9. Integumentary guanine content, classically associated with thyroid activity did not significantly change between 0.68 to 0.85 $\mu mole/cm^2$ and could not be related to increased plasma T_3 (5.0 to 8.4 to 45 $\mu mole/ml$) but a trend appeared to be developing.

10. Increased plasma T_3 effects increased occupancy of saturable nuclear T_3 binding sites but is associated with both increased or decreased Na^+/K^+-ATPase activity in two different tissues.

LITERATURE CITED

Adams, B.L., Zaugg, W.S., and McLain, L.R. (1973). Temperature effect on parr-smolt transformation in steelhead trout (Salmo gairdneri) as measured by gill sodium-potassium stimulated adenosine triphosphatase. Comp. Biochem Physiol. 44A, 1333-1339.

Adams, B.L., Zaugg, W.S., and McLain, L.R. (1975). Inhibition of salt water survival and Na-K-ATPase elevation in steelhead trout (Salmo gairdneri) by moderate water temperatures. Trans. Amer. Fish. Soc. 104, 766-769.

Apriletti, J.W., Eberhardt, N.L., Latham, K.R., and Baxter, J.D. (1981). Affinity chromatography of thyroid hormone receptors. J. Biol. Chem. 256, 12094-12101.

Asano, Y., Liberman, U.A., and Edelman, I.S. (1976). Thyroid thermogenosis. J. Clin. Invest. 57, 368-379.

Baggerman, B. (1960). Salinity preference, thyroid activity and the seaward migration of four species of Pacific Salmon (Oncorhynchus). J. Fish. Res. Bd. Can. 17, 295-322.

Ball, J.N., Olivereau, M., and Kollman, K.D. (1963). Secretion of thyrotrophic hormone by pituitary transplants in a teleost fish. Nature 199, 618-620.

Baxter, J.D., Eberhardt, N.L., Apriletti, J.W., Johnson, L.K., Ivarie, R.D., Schachter, B.S., Morris, J.A. Seeburg, P.H., Goodman, H.M., Latham, K.R. Polansky, J.R., and Martial, J.A. (1975). Thyroid hormone receptors and responses. Rec. Prog. Horm. Res. 35, 97-147.

Bellabarba, D., and Lehoux, J.G. (1981). Triiodothyronine nuclear receptor in chick embryo: nature and properties of the hepatic receptor. Endocrinology 109, 1017-1025.

Bernal, J., and DeGroot, L.J. (1977). Thyroid hormone receptors: release of receptor to the medium during in vitro incubation of isolated rat liver nuclei. Endocrinology 100, 648-655.

Bhattacharya, A., and Vonderhaar, B.K. (1977). Specific binding proteins for 3,5,3'-triiodothyronine in mouse mammary epithelium. J. Cell. Biol. 75, 47A.

Blobel, G., and Potter, V.R. (1966). Nuclei from rat liver: isolation method that combines purity from high yield. Science 154, 1662-1665.

Brown, S.B., and Eales, J.G. (1977). Measurement of L-thyroxine and 3,5,3'-triiodo-L-thyronine levels in fish plasma by radioimmunoassay. Can. J. Zool. 55, 293-299.

Buergi, U., and Larsen, P.R. (1982). Nuclear triiodothyronine binding in mononuclear leukocytes in normal subjects and obese patients before and after fasting. J. Clin. Endocrinol. Metabol. 54, 1199-1205.

Burke, R.E., and McGuire, W.L. (1978). Nuclear thyroid hormone receptors in a human breast cancer cell line. Cancer Res. 38, 3769-3773.

Burman, K.D., Latham, K.R., Djuh, Y.Y., Smallridge, R.C.,
Tseng, Y.C.L., Lukes, Y.G., Maunder, R., and
Wartofsky, L. (1980). Solubilized nuclear thyroid
hormone receptors in circulating human mononuclear
cells. J. Clin. Endocrinol. Metabol. 51, 106-116.

Burman, K.D., Lukes, Y., Wright, F.D., and Wartofsky, L.
(1977). Reduction in hepatic triiodothyronine binding
capacity induced by fasting. Endocrinology 101, 1331-1334.

Burton, K. (1956). A study of the conditions and mechanism
of the diphenylamine reactions for the colorimetric
estimation of deoxyribonucleic acid. Biochemistry
62, 315-322.

Butler, D.G., and Carmichael, F.J. (1972). (Na^+-K^+)-ATPase
activity in eel (Anguilla rostrata) gills in relation
to changes in environmental salinity: role of
adrenocortical steriods. Gen. Comp. Endocrinol. 19,
421-427.

Carilli, C.T., Farley, R.A., Perlman, D.M., and Cantley, L.C.
(1982). The active site structure of Na^+- and K^+-
stimulated ATPase. J. Biol. Chem. 257, 5601-5606.

Cheng, S.Y., Maxfield, F.R., Robbins, J., Willingham, M.C.,
and Pastan, I.H. (1980). Receptor-mediated uptake
of 3,3',5'-triiodo-L-thyronine by cultured fibroblasts.
Proc. Nat'l. Acad. Sci. 6, 3425-3429.

Chua, D., and Eales, J.G. (1971). Thyroid and dermal
purines in brook trout, Salvelinus fontinalis (Mitchill)
Can. J. Zool. 49, 1557-1561.

Clarke, W.C., Folmar, L., and Dickhoff, W.W. (1978).

Changes in gill Na^+-K^+ ATPase, plasma sodium and

thyroid hormone levels during adaptation of juvenile

coho salmon to sea water. Amer. Zool. 18, 652.

Coulombe, P., Ruel, J., and Dussault, J.H. (1979).

Analysis of nuclear 3,5,3'-triiodothyronine-binding

capacity and tissue response in the liver of the

neonatal rat. Endocrinology 105, 952-959.

Crantz, F.R., Silva, J.E., and Larsen, P.R. (1982).

An analysis of the sources and quantity of

3,5,3'-triiodothyronine specifically bound to nuclear

receptors in rat cerebral cortex and cerebellum.

Endocrinology 110, 367-375.

Darling, D.S., Dickhoff, W.W., and Gorbman, A. (1980).

Nuclear thyroid hormone receptors in salmon. Amer.

Zool. 20, 858.

Darling, D.S., Dickhoff, W.W., and Gorbman, A. 1982.

Comparison of thyroid hormone binding to hepatic

nuclei of the rat and a teleost (Oncorhynchus kisutch).

Endocrinology 111, 1936-1943.

Das, D.K., and Ganguly, M. (1981). Diabetes, hypophysectomy

or thyroidectomy reduces nuclear L-triiodothyronine-

binding capacity of rat lung. Endocrinology 109,

296-300.

DeGroot, L.J., Hill, L., and Rue, P. (1976a). Binding of
    nuclear triiodothyronine (T_3) binding protein-T_3
    complex to chromatin. Endocrinology 99, 1605-1611.

DeGroot, L.J., Refetoff, S., Strausser, J., and Barsano, C.
    (1974). Nuclear triiodothyronine-binding protein:
    partial characterization. Proc. Nat'l. Acad. Sci.
    71, 4042-4046.

DeGroot, L.J., and Strausser, J.L. (1974). Binding of T_3
    in rat liver nuclei. Endocrinology 95, 74-83.

DeGroot, L.J., and Torresani, J. (1975). Triiodothyronine
    binding to isolated liver cell nuclei. Endocrinology
    96, 357-369.

DeGroot, L.J., Toressani, J., Carrayon, P., and Tirard, A.
    (1976b). Factors influencing triiodothyronine binding
    properties of liver nuclear receptors. Acta
    Endocrinol. 83, 293-304.

Dickhoff, W. W., Darling, D.S., and Gorbman, A. (1982).
    Thyroid function during smoltification of salmonid
    fish. in "Phylogenic aspects of thyroid hormone
    action." (S. Suzuki ed.). Center for Academic
    Publications Japan, Tokyo.

Dickhoff, W.W., Folmar, L., and Gorbman, A. (1977).
    Relationship of thyroxine and gill Na^+-K^+
    adenosinetriphosphatase (ATPase) in coho salmon,
    Oncorhynchus kisutch. Amer. Zool. 17, 857.

Dickhoff, W.W., Folmar, L.C., and Gorbman, A. (1978a).
Changes in plasma thyroxine during smoltification
in coho salmon, Oncorhynchus kisutch. Gen. Comp.
Endocrinol. 36, 229-232.

Dickhoff, W.W., Folmar, L.C., and Gorbman, A. (1978b).
Changes in thyroid hormones during the early
development of coho salmon (Oncorhynchus kisutch).
Amer. Zool. 18, 652.

Dillman, W.H., Bonner, R.A., and Oppenheimer, J.H. (1978a).
Glucagon administration decreases hepatic nuclear
triiodothyronine binding capacity. Endocrinology
102, 1633-1636.

Dillman, W.H., Schwartz, H.L., and Oppenheimer, J.H. (1978b).
Selective alterations in hepatic enzyme response
after reduction by nuclear triiodothyronine receptor
sites by partial hepatectomy and starvation. Biochem.
Biophys. Res. Comm. 80, 259-266.

Eales, J.G. (1969). A comparative study of purines
responsible for silvering in several freshwater fish.
J. Fish. Res. Bd. Can. 26, 1927-1931.

Eales, J.G. (1974). Creation of chronic physiological
elevations of plasma thyroxine in brook trout,
Salvelinus fontinalis (Mitchill) and other teleosts.
Gen. Comp. Endocrinol. 22, 209-217.

Eales, J.G. (1979a). Thyroid function in cyclostomes and fishes. in "Hormones and Evolution" vol. 1. (E.J.W. Barrington, ed.), pp. 342-436. Academic Press, New York.

Eales, J.G. (1979b). Comparison of L-thyroxine and 3,5,3'triiodo-L-thyronine kinetics in fed and starved rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol. 62A, 295-300.

Eales, J.G., and Collicut, J.M. (1974). Uptake of ambient radioactive L-thyroxine and 3,5,3'-triiodo-L-thyronine by rainbow trout, Salmo gairdneri (Richardson). Gen. Comp. Endocrinol. 24, 347-351.

Eales, J.G., Van der Kraak, G.J., Chang, J.P., Omeljaniuk, R.J., and Uin, L. (1983). Effect of temperature on thyroid hormone kinetics in rainbow trout, Salmo gairdneri, (Richardson). Gen. Comp. Endocrinol. (in preparation).

Eberhardt, N.L., Valcana, T., and Timiras, P.S. (1976). Hormone-receptor interactions in brains: uptake and binding of thyroid hormone. Psychoneuroendocrinology 1, 399-409.

Eberhardt, N.L, Valcana, T., and Timiras, P.S. (1978). Triiodothyronine nuclear receptors: an in vitro comparison of the binding of triiodothyronine to nuclei of adult rat liver, cerebral hemisphere, and anterior pituitary. Endocrinology 102,556-561.

Eckel, J., Rao, G.S., Rao, M.L., and Bruer, M. (1979).
Uptake of L-tri-iodothyronine by isolated rat liver
cells. Biochem. J. 182, 473-491.

Etkin, W. (1935). The mechanisms of anuran metamorphosis
pattern. J. Expt'l. Zool. 71, 317-340.

Etkin, W. (1968). Hormonal control of amphibian metamorphosis.
in "Metamorphosis, a problem in developmental biology."
(H. Etkin and L.I. Gilbert, eds), pp. 313-348.
Appleton-Century-Crofts, New York.

Evans, D.H. and Mallery, C.M. (1975). Time course of
sea water acclimation by the euryhaline teleost,
Dormitator maculatus. J. Comp. Physiol. 96, 117-122.

Ewing, R.D., Johnson, S.L., Pribble, H.J., and Lichatowich,
J.A. (1979). Temperature and photoperiod effects on
gill (Na^+K)- ATPase activity in chinook salmon
(Oncorhynchus tshawytscha). J. Fish. Res. Bd. Can.
36, 1347-1353.

Fiske, C.H. and Subbarow, Y. (1925). The colorimetric
determination of phosphorous. J. Biol. Chem. 66,
375-400.

Folmar, L.C. and Dickhoff, W.W. (1978). Some physiological
changes during smoltification of coho salmon
(Oncorhynchus kisutch). Amer. Zool. 18, 606.

Folmar, L.C., and Dickhoff, W.W. (1979). Plasma thyroxine and gill Na^+-K^+-ATPase changes during seawater acclimation of coho salmon, Oncorhynchus kisutch. Comp. Biochem. Physiol. 63A, 329-332.

Galton, V.A. 1980. Binding of thyroid hormones in vivo by hepatic nuclei of Rana catesbiana tadpoles. Endocrinology 106, 859-866.

Gardner, R.S. (1978). Nuclear thyroid hormone receptor binding to chromatin subunits: implications from digestion studies with micrococcal nuclease. Molec. Cell. Endocrinol. 10, 277-292.

Garg, L.C., Mackie, S., and Tisher, C.C. (1982). Site of action of thyroid hormone on Na-K-ATPase in rat nephron segments. Kidney Int. 21, 274.

Geering, K., Girardet, M., Bron, C., Kraehenbühl, J., and Rossier, B.C. (1982). Hormonal regulation of (Na^+, K^+)-ATPase biosynthesis in the toad bladder. J. Biol. Chem. 257, 10338-10343.

Geering, K., and Rossier, B.C. (1980). Characterization of high affinity nuclear binding sites for triiodothyronine (T_3) in an adult amphibian. Experimentia 36, 745.

Geering, K., and Rossier, B.C. (1981). Thyroid hormone - aldosterone antagonism on Na^+ transport in toad bladder. J. Biol. Chem. 256, 5504-5510.

Giles, M.A., and Vanstone, W.E. (1976). Changes in
oubain-sensitive adenosine triphosphatase activity
in gills of coho salmon (Oncorhynchus kisutch)
during parr-smolt transformation. J. Fish. Res.
Bd. Can. 33, 54-62.

Gonzales, L.K., and Ballard, P.L. (1981). Characterization
of triiodothyronine (T_3)-binding sites in fetal
human lung nuclei. Fed. Proc. 40, 592.

Gonzales, L.K., and Ballard, P.L. (1982). Nuclear
3,5,3'-triiodothyronine receptors in rabbit lung:
characterization and developmental changes.
Endocrinology 111, 542-552.

Gordon, A., and Spira, O. (1975). Triiodothyronine
binding in rat anterior pituitary, posterior
pituitary, median eminence and brain. Endocrinology
96, 1357-1365.

Groul, D.J. (1977). Cooperative binding of thyroid hormone
to chromatin receptors. J. Cell Biol. 75, 136A.

Groul, D.J. (1980a). 3,5,3'-triiodothyronine receptor-
containing chromatin fragments: production by
nuclease digestion. Endocrinology 107, 994-999.

Groul, D.J. (1980b). An assay for thyroid hormone binding
to chromatin receptors. Anal. Biochem. 101, 387-393.

Groul, D.J., and Kempner, E.S. (1982). The size of
the thyroid hormone receptor in chromatin. J.
Biol. Chem. 257, 708-713.

Haber, R.S., and Loeb, J.N. (1982). Effect of
3,5,3'-triiodothyronine treatment on potassium
efflux from isolated rat diaphragm: role of increased
permeability in the thermogenic response.
Endocrinology 111, 1217-1223.

Halpern, J., and Hinkle, P.M. (1982). Evidence for an
active step in thyroid hormone transport to
nuclei: drug inhibition of L-^{125}I-triiodothyronine
binding to nuclear receptors in rat pituitary
tumour cells. Endocrinology 110, 1070-1072.

Hamada, S., Nakamura, H., Nanno, M., and Imura, H. (1979).
Triiodothyronine-induced increase in rat liver
nuclear thyroid-hormone receptors associated with
increased mitochondrial α-glycerophosphate
dehydrogenase activity. Biochem. J. 182, 371-375.

Hawk, P.B., Oser, B.L., and Summerson, W.H. (1954).
Practical physiological chemistry. 13th ed. J.A.
Churchill, Ltd., London.

Higgs, D.A. and Eales, J.G. (1971). Iodine and thyroxine
metabolism in the brook trout, Salvelinus fontinalis
(Mitchill), during sustained exercises. Can. J.
Zool. 49, 1255-1269.

Hitchings, G.H. and Falco, E.A. (1944). The identification of guanine in extracts of Ginella nigricans. The specificity of guanase. Proc. Nat'l. Acad. Sci. 30, 294-297.

Hoar, W.S. (1939). The thyroid gland of the Atlantic salmon. J. Morphol. 65, 257-295.

Hoar, W.S., and Bell, G.M. (1950). The thyroid gland in relation to the seaward migration of Pacific salmon. Can. J. Res. D. 28, 126-136.

Hoar, W.S., Black, Y.S., and Black, E.C. (1951). Hormones in fish. Some aspects of the physiology of fishes. Univ. Toronto Biol. Ser. 59, 1-111.

Honn, K.V., and Chavin, W. (1977). Mechanism of T_4 action upon nurse shark gill and kidney tissues. Amer. Zool. 17, 857.

Horiuchi, R., Cheng, S., Willingham, M., and Pasten, I. (1982). Inhibition of the nuclear entry of 3,3',5-triiodo-L-thyronine by monodansylcadavorine in GH-3 cells. J. Biol. Chem. 257, 3139-3144.

Hunt, D.W.C., and Eales, J.G. (1979). The influence of testosterone propionate on thryoid function of immature rainbow trout, Salmo gairdneri Richardson. Gen. Comp. Endocrinol. 37, 115-121.

Inoue, A., Nakagawa, K., and Morisawa, S. (1981). Effect of DNA on thyroid hormone binding by specific receptor proteins from rat liver nuclei. Eur. J. Biochem. 114, 509-516.

Ismail-Beigi, F., Bissell, D.M., and Edelman, I.S. (1979). Thyroid thermogenesis in adult rat hepatocytes in primary monolayer culture. J. Gen. Physiol. 73, 369-383.

Ismail-Beigi, F., and Edelman, I.S. (1970). Mechanism of thyroid calorigenesis: role of active sodium transport. Proc. Nat'l. Acad. Sci. 67, 1071-1978.

Ismail-Beigi, F., and Edelman, I.S. (1971). The mechanism of the calorigenic action of thyroid hormone: stimulation of the Na^++K^+-activated adenosinetriphosphatase activity. J. Gen. Physiol. 57, 710-722.

Ismail-Beigi, F., and Edelman, I.S. (1973). Effects of thyroid status on electrolyte distribution in rat tissues. Amer. J. Physiol. 225, 1172-1177.

Ismail-Beigi, F., and Edelman, I.S. (1974a). Time course of the effects of thyroid hormone on respiration and Na^++K^+-ATPase in rat liver. Proc. Soc. Expt'l. Biol. Med. 146, 983-988.

Ismail-Beigi, F., and Edelman, I.S. (1974b). Thyroid thermogenesis and active sodium transport. Rec. Prog. Horm. Res. 30, 235-257.

Jaffe, R.C. and Means, A.R. (1977). Stability of nuclear triiodothyronine binding sites. Endocrinology 101, 447-452.

Jampol, L.M., and Epstein, F.H. (1970). Sodium-potassium-activated adenosine triphosphatase and osmotic regulation by fishes. Amer. J. Physiol. 218, 607-611.

Johnson, D.W. (1973). Endocrine control of hydromineral balance in teleosts. Amer. Zool. 13, 799-818.

Johnson, S.L., Ewing, R.D., and Lichatowich, J.A. (1977). Characterization of gill (Na^+K^+)-activated adenosine triphosphatase from chinook salmon, Oncorhynchus tshawytscha. J. Expt'l. Zool. 199, 345-354.

Johnston, C.E., and Eales, J.G. (1967). Purines in the integument of the Atlantic salmon (Salmo salar) during parr-smolt transformation. J. Fish. Res. Bd. Can. 24, 955-964.

Jorgensen, P.L. (1974). Purification of (Na^++K^+)-ATPase: active site determinations and criteria of purity. Annal. N.Y. Acad. Sci. 242,36-52.

Jorgensen, P.L, and Skou, J.C. (1969). Preparation of highly active (Na^++K^+)-ATPase from the outer medulla of rabbit kidney. Biochem. Biophys.Res. Comm. 37, 39-46.

Jump, D.B., Seelig, S., Schwartz, H.L., and Oppenheimer, J.H. (1981). Association of the thyroid hormone receptor with rat liver chromatin. Biochemistry. 20, 6781-6789.

Kaltenbach, J.C. (1982). Circulating thyroid hormone levels in amphibia. in Phylogenetic aspects of thyroid hormone action." (S. Suzuki, ed.) pp. 63-74. Center for Academic Publications Japan, Tokyo.

Kamiya, M., and Utida, S. (1968). Changes in activity of sodium-potassium-activated adenosinetriphosphatase in gills during adaptation of the japanese eel to sea water. Comp. Biochem. Physiol. 26, 675-685.

Kamiya, M., and Utida, S. (1969). Sodium-potassium activated adenosinetriphosphatase activity in gills of fresh-water, marine, and euryhaline teleosts. Comp. Biochem. Physiol. 31, 671-674.

Kirschner, L.B. (1969). ATPase activity in gills of euryhaline fish. Comp. Biochem. Physiol. 29, 871-874.

Kistler, A., Yoshizato, K., and Frieden, E. (1975). Binding of thyroxine and triiodothyronine by nuclei of isolated tadpole liver cells. Endocrinology 97, 1036-1042.

Klein, A.H., Jenkins, J., Reviczky, A., and Fisher, D.A. (1981). Dissociation of thyroid hormone stimulated respiration and Na/K-ATPase activity in brown adipose tissue (BAT) and liver in the newborn rabbit. Pediatric. Res. 15, 483.

Klotz, I.M. (1982). Number of receptor sites from
    Scatchard graphs: facts and fantasies. Science
    217, 1247-1249.

Koerner, D., Surks, M.I., and Oppenheimer, J.H. (1974).
    In vitro demonstration of specific triiodothyronine
    binding sites in rat liver nuclei. J. Clin.
    Endocrinol. Metabol. 38, 706-709.

Krenning, E.P., Docter, R., Bernard, H.F., Visser, T.J.,
    and Hennemann, G. (1978). Active transport of
    triiodothyronine (T_3) into isolated rat liver cells.
    FEBS. Letters 91, 113-116.

Krenning, E., Docter, R., Bernard, B., Visser, T., and
    Hennemann, G. (1980). Regulation of the active
    transport of 3,3',5-triiodothyronine (T_3) into
    primary cultured rat hepatocytes by ATP. FEBS.
    Letters 119, 279-282.

Krenning, E.P., Docter, R., Bernard, B., Visser, T., and
    Henneman, G. (1982). Decreased transport of
    thyroxine (T_3), 3,3',5'triiodothyronine (T_3), and
    3,3',5'-triiodothyronine (rT_3) into rat hepatocytes
    in primary culture due to a decrease of cellular ATP
    content and various drugs. FEBS. Letters. 140, 229-233.

Krug, E.C., Honn, K.V., Battista, J., and Nicoll, C.S. (1980).
    Effects of thyroxine (T_4) and ACTH treatment on
    corticosteroid levels in serum of bullfrog tadpoles.
    Amer. Zool. 20, 858.

Landgrebe, J.S. (1941). The role of the pituitary and the thyroid in the development of teleosts. J. Expt'l. Biol. 18, 162-169.

LaRoche, G., and Leblond, C.P. (1952). Effect of thyroid preparations and iodide on salmonidae. Endocrinology 51, 524-545.

Latham, K.R., Apriletti, J.W., Eberhardt, N.L., and Baxter, J.D. (1981). Development of support matrices for affinity chromatography of thyroid hormone receptors. J. Biol. Chem. 256, 12088-12093.

Latham, K.R., Ring, J.C., Baxter, J.D. (1976). Solubilized nuclear receptors for thyroid hormones. Physical characteristics and binding properties, evidence for multiple forms. J. Biol. Chem. 251, 7388-7397.

Las, M.S., and Surks, M.I. (1981). Dissociation of serum triiodothyronine concentration and hepatic nuclear triiodothyronine-binding capacity in streptozotocin-induced diabetic rats. Endocrinology 109, 1259-1263.

Layden, T.J., and Boyer, J.L. (1976). The effect of thyroid hormone on bile salt-independent bile flow and Na^+, K^+-ATPase activity in liver plasma membranes enriched in bile canaliculi. J. Clin. Invest. 57, 1009-1018.

Liberman, U.A., Asano, Y., and Edelman, I.S. (1976).
Role of active sodium transport in small intestinal
mucosa in thyroid thermogenesis. Israel J. Med.
Sci. 12, 1519.

Liberman, U.A., Asano, Y., Lo, C.S., and Edelman, I.S.
(1979). Relationship between Na^+-dependent
respiration and $Na^+ + K^+$-adenosine triphosphatase
activity in the action of thyroid hormone on rat
jejeunal mucosa. Biophys. J. 27, 127-144.

Lin, M.H., and Akera, T. (1978). Increased (Na^+, K^+)-ATPase
concentrations in various tissues of rats caused
by thyroid hormone treatment. J. Biol. Chem.
253, 723-726.

Lindenberg, J.A., Brehier, A., and Ballard, P.L. (1978).
Triiodothyronine nuclear binding in fetal and adult
rabbit lung and cultured lung cells. Endocrinology
103, 1725-1731.

Lo, C.S., Cheng, W., and Klein, L.E. (1981a). Effect of
triiodothyronine on $(Na^+ + K^+)$-adenosine triphosphatase
and $(Na^+$ and $Mg^{++})$-dependent phosphorylated
intermediate in rat salivary glands. Pflugers
Arch. 392, 134-138.

Lo, C.S., Cheng, W., and Klein, L. (1981b). Effect of
triiodothyronine on $(Na^+ + K^+)$ adenosine triphosphatase
in rat salivary gland. Fed. Proc. 40, 613.

Lo, C.S., August, T.R., Liberman, U.A., and Edelman, I.S. (1976). Dependence of renal ($Na^+ + K^+$)-adenosine triphosphatase activity on thyroid status. J. Biol. Chem. 251, 7826-7833.

Lo, C.S., and Edelman, I.S. (1976). Effect of triiodothyronine on the synthesis and degradation of renal cortical ($Na^+ + K^+$)-adenosine triphosphatase. J. Biol. Chem. 251, 7834-7840.

Lo, C.S., and Lo, T.N. (1981). Triiodothyronine augments the number of membrane-bound ($Na^+ - K^+$)-adenosine triphosphatase units, but does not affect the senimentation properties of plasma membrane components. Endocrinology 109, 1473-1478.

Maberly, G.F., Eastman, C.J., Smith, H.C. Waite, K., and Wilke, T.J. (1982). Thyroid hormone binding to putative nuclear receptors in human mononuclear blood cell. Austral. J. Expt'l. Biol. Med. Sci. 60, 113-115.

Martial, J.A., Seeburg, P.H., Guenzi, D., Goodman, H.M., and Baxter, J.D. (1977a). Regulation of growth hormone gene expression: synergistic effects of thyroid and glucorticoid hormones. Proc. Nat'l. Acad. Sci. 74, 4293-4298.

Markert, J.R., and Vanstone, W.E. (1966). Pigments in
the belly skin of coho salmon, Oncorhynchus kistuch.
J. Fish. Res. Bd. Can. 23, 1095-1098.

Mashio, Y., Inada, M., Tanaka, K., Sshii, H., Naito, K.,
Nishikawa, M., Takahaski, K., and Imura, J.G. (1982).
High affinity 3,5,3'-L-triiodothyronine binding
to synaptosomes in rat cerebral cortex.
Endocrinology 110, 1257-1261.

Massey, B.S., and Smith, C.L., (1968). The action of thyroxine
on mitochondrial respiration and phosphorylation in
the trout (Salmo trutta fario L.). Comp. Biochem.
Physiol. 25, 241-255.

Matty, A.J., and Green, K. (1964). Effect of thyroxine
on ion movement in isolated toad bladder. Gen.
Comp. Endocrinol. 4, 331-338.

Matty, A.J., and Sheltawy, M.J. (1967). The relation
of thyroxine to skin purines in Salmo irideus. Gen.
Comp. Endocrinol. 9, 473.

McCartney, T.H. (1976). Sodium-potassium dependent
adenosine triphosphatase in gills and kidneys
of Atlantic salmon (Salmo salar). Comp. Biochem.
Physiol. 53A, 351-353.

McCarty, L.S., and Houston, A.H. (1977). Na^+:K^+-and
HCO_3^-- stimulated ATPase activities in the gills
and kidneys of thermally acclimated rainbow trout
(Salmo gairdneri). Can. J. Zool. 55, 704-712.

Meitus, L.J., and Pardridge, W.M. (1980). Carrier-mediated transport of thyroid hormones through the blood-brain barrier of the newborn rabbit. Fed. Proc. 39, 732.

Menezes-Ferreira, M.M.M., Bismuth, J., and Torresani, J. (1982). Reversible dissociation of triiodothyronine-nuclear receptor complexes by mercurial and chaotropic reagents. Biochem. Biophys. Res. Comm. 105, 244-251.

Morishige, W.K., and Geurnsey, D.L. (1978). Triiodothyronine receptors in rat lung. Endocrinology 102, 1628-1632.

Murthy, P.V.N., Banovac, K., and McKenzie, J.M. (1978). Hypothyroidiom-induced changes in triiodothyronine binding to nuclei and cytosol binding proteins in rat liver. Endocrinology 102, 1129-1136.

Nakamura, H., Hamada, S., and Imura, H. (1979). Increase in liver nuclear triiodothyronine receptors associated with increased deoxyribonucleic acid by longterm administration of triiodothyronine in thyroidectomized rats. Biochem. J. 184, 143-148.

Okamura, K., Taurog, A., and DiStefano, J.J. (1981). Elevated serum levels of T_3 without metabolic effect in nutritionally deficient rats, attributable to reduced cellular uptake of T_3. Endocrinology 109, 673-675.

Oppenheimer, J.H., Koerner, D., Schwartz, H.L., and
Surks, M.I. (1972). Specific nuclear triiodothyronine
binding sites in rat liver and kidney. J. Clin.
Endocrinol. Metabol. 35, 330-333.

Oppenheimer, J.H., Schwartz, H.L., Koerner, D. and Surks, M.I.
(1974a). Limited binding capacity sites for
L-triiodothyronine in rat liver nuclei. Nuclear-
cytoplasmic interrelation, binding constants, and
cross-reactivity with L-thyroxine. J. Clin. Invest.
53, 768-777.

Oppenheimer, J.H., Schwartz, H.L., and Surks, M.I. (1974b).
Tissue differences in the concentration of
triiodothyronine nuclear binding sites in the rat:
liver, kidney, pituitary, heart, brain, spleen, and
testis. Endocrinology 95, 897-903.

Oppenheimer, J.H., Silva, E., Schwartz, H.L., and Surks,
M.I. (1977). Stimulation of hepatic mitochondrial
α-glycerophosphate dehydrogenase and malic enzyme by
L-triiodothyronine. J. Clin. Invest. 59,517-521.

Pardridge, W.W., and Meitus, L.J. (1980). Transport of
thyroid and steroid hormones through the blood brain
barrier of the newborn rabbit: primary role of
protein bound hormone. Endocrinology 107, 1705-1710.

Pasqual, A., Casanova, J., and Samuels, H.H. (1982).
Photoaffinity labelling the thyroid hormone nuclear
receptors in intact cells. J. Biol. Chem. 257,
9640-9647.

Perlman, A.J., Stanley, F.M., Pasqual, A., and Samuels,
H.H. (1981). Interaction of the thyroid hormone
receptor with chromatin components. Clin. Res.
29, 567.

Perlman, A.J. Stanley, F., and Samuels, H.H. (1982).
Thyroid hormone nuclear receptor. J. Biol. Chem.
257, 930-938.

Peschen, K.E. (1939). Untersuchungen uber das Vorkommen
und den Stoffwechsel des Guanins im Tierreich.
Zool. Jahrb. Abt. Allgem. Zool. Physiol. Tierre.
59, 429-459.

Pettine, K. (1981). Direct thyroid hormone stimulation
of NaK-ATPase in cultured hepatocyts. Clin. Res.
29, 86A.

Phillipson, K.D., and Edelman, I.S. (1977a). Thyroid
hormone control of Na^+-K^+-ATPase and K^+-dependent
phosphatase in rat heart. Amer. J. Physiol. 232,
C196-201.

Phillipson, K.D., and Edelman, I.S. (1977b).
Characteristics of thyroid stimulated Na^+-K^+-ATPase
of rat heart. Amer. J. Physiol. 232, C202-206.

Premdas, F.H., and Eales, J.G. (1976a). Identification of pteridines and purines in the skin of rainbow trout (Salmo gairdneri) and brook trout (Salvelinus fontinalis). Comp. Biochem. Physiol. 53B, 561-567.

Premdas, F.H., and Eales, J.G. (1976b). The influence of TSH and ACTH on purine and pteridine deposition in the skin of rainbow trout (Salmo gairdneri). Can. J. Zool. 54, 576-581.

Raaka, B.M., and Samuels, H.H. (1981). Regulation of thyroid hormone receptor levels in GH-1 cells by 3,5,3'-triiodo-L-thyronine. Use of dense amino acid labelling to determine the influence of hormone on the receptor half-life and the rate of appearance of newly synthesized receptor. J. Biol. Chem. 256, 6883-6889.

Rao, G.S. (1981). Mode of entry of steroid and thyroid hormones into cells. Molec. Cell. Endocrinol. 21, 97-108.

Rao, G.S., Eckel, J., Rao, M.L., and Breuer, H. (1976a). Uptake of thyroid hormone by isolated rat liver cells. Biochem. Biophys. Res. Comm. 73, 98-104.

Rao, G.S., Eckel, J., Rao, M.L., and Breuer, H. (1976b). Evidence to show that uptake of L-triiodothyronine by liver cells is consistent with transport of the hormone by a carrier mediated process. Annal. d'Endocrinol. 40, 37A.

Robertson, O.H. (1948). The occurrence of increased

activity of the thyroid gland in rainbow trout at

the same time of transformation from parr to

silvery smolt. Physiol. Zool. 21, 282-294.

Robertson, O.H. (1949). Production of the silvery smolt

stage in rainbow trout by intramuscular injection

of mammalian thyroid extract and thyrotropic hormone.

J. Expt'l. Zool. 110,337-355.

Rodbard, D. (1973). Mathematics of hormone-receptor

interaction. I Basic principals. In "Receptors

for Reproductive Hormones" (B.H. O'Malley and

A.R. Means, eds.), pp. 289-326. Plenum, New York.

Rodriguez-Pena, A., and Bernal, J. (1982a). Thyroid

hormone dependent nuclear proteins from rat liver.

Acta. Endocrinol. 99, 567-572.

Rodiguez-Pena, A., and Bernal. J. (1982b). Effect of

divalent cations on the binding of 3,5,3'-

triiodothyronine to isolated rat liver nuclei.

Endocrinology 110,246-253.

Rossier, B.C., Gaggeler, H.P., and Rossier, M. (1978).

Thyroid hormone inhibition of the late aldosterone

response in the urinary bladder of the toad Bufo

marinus. Experientia. 34, 929.

Ruel, J., Coulombe, P., and Dussault, J.H.   (1982).
   Characterization of nuclear 3,5,3'-triiodothyronine
   receptors in the developing rat lung: effects of
   hypo- and hyperthyroidism. Pediatr. Res., 16,
   238-242.

Samuels, H.H., Perlman, A.J., Raaka, B.M., and Stanley, F.
   (1982). Organization of the thyroid hormone receptor
   in chromatin. Rec. Prog. Horm. Res. 38, 557-592.

Samuels, H.H. and Shapiro, L.E. (1976). Thyroid hormone
   stimulates denovo growth hormone synthesis in
   cultured GH-1 cells: evidence for the accumulation
   of a rate limiting RNA species in the induction
   process. Proc. Nat'l. Acad. Sci. 73, 3369-3373.

Samuels, H.H., Stanley, F., and Shapiro, L.E. (1977).
   Modulation of thyroid hormone nuclear receptor levels
   by 3,5,3'-triiodo-L-thyronine in GH-1 cells. Evidence
   for two functional components of nuclear-bound
   receptor and relationship to the induction of growth
   hormone synthesis. J. Biol. Chem. 252, 6052-6060.

Samuels, H.H., and Tsai, J.S. (1973). Thyroid hormone
   action in cell culture: demonstration of nuclear
   receptors in intact cells and isolated nuclei.
   Proc. Nat'l. Acad. Sci. 70, 3488-3492.

Samuels, H.H., and Tsai, J.S. (1974). Thyroid hormone
   actions demonstration of similar receptors in isolated
   nuclei of rat liver and cultured GH, cells. J. Clin.
   Invest. 53, 656-659.

Samuels, H.H., Tsai, J.S., and Casanova, J. (1974a).
Thyroid hormone action: in vitro demonstration of
putative receptors in isolated nuclei and soluble
nuclear extracts. Science 184, 1188-1191.

Samuels, H.H., Tsai, J.S., Casanova, J., and Stanley, F.
(1974b). Thyroid hormone action: in vitro
characterization of solubilized nuclear receptors
from rat liver and GH-1 cells. J. Clin. Invest.
54, 853-865.

Saunders, R.L., and Henderson, E.B. (1978). Changes in
gill ATPase activity and smolt status of Altantic
salmon (Salmo salar). J. Fish. Res. Bd. Can. 35,
1542-1546.

Scatchard, G. (1949). The attractions of proteins for
small molecules and ions. Annal. N.Y. Acad. Sci.
51, 660-672.

Schadlow, A.R., Surks, M.I. Schwartz, H.L., and
Oppenheimer, J.H. (1972). Specific triiodothyronine
binding sites in the anterior pituitary of the rat.
Science 176, 1252-1254.

Schussler, G.C., and Orlando, J. (1978). Fasting decreases
triiodothyronine receptor complex. Science 199,
686-688.

Schuster, L.D., Schwartz, H.L., and Oppenheimer, J.H.
(1979). Nuclear receptors for 3,5,3'-triiodothyronine
in human liver and kidney: characterization,
quantitation and similarities to rat receptors.
J. Clin. Endocrinol. Metabol. 48, 627-632.

Schwartz, H.L., and Oppenheimer, J.H. (1978b). Nuclear
triiodothyronine receptor sites in brain: probable
identity with hepatic receptors and regional
distribution. Endocrinology 103, 267-273.

Sedmak, J.J., and Grossberg, S.E. (1977). A rapid,
sensitive, and versatile assay for protein using
Coomassie Brilliant Blue G-250. Anal. Biochem.
79, 544-552.

Seelig, S., Liaw, C., Towle, H.C. and Oppenheimer, J.H.
(1981a). Thyroid hormone attenuates and augments
hepatic gene expression at a pretranslational level.
Proc. Nat'l. Acad. Sci. 78, 4733-4737.

Seelig, S., Schwartz, H.L., and Oppenheimer, J.H. (1981b).
Limitations in the conventional analysis of the
interaction of triiodothyronine with solubilized
nuclear receptor sites. J. Biol. Chem. 256, 2154-2161.

Shimomura, Y., Lee, M., Oku, J., Bray, G.A., and Glick, Z.
(1982). Sodium potassium dependent ATPase in
hypophysectomized rats: response to growth hormone,
triiodothyronine, and cortisone. Metabolism 31, 213-216.

Silva, J.E., and Larsen, P.R. (1978). Contributions of plasma triiodothyronine and local thyroxine monodeiodination to triiodothyronine to nuclear triiodothyronine receptor saturation in pituitary, liver, and kidney of hypothyroid rats. J. Clin. Invest. 61, 1247-1259.

Silva, E.S., Astier, H., Thakare, U., Schwartz, H.L. and Oppenheimer, J.H. (1977). Partial purification of the triiodothyronine receptor from rat liver nuclei. Differences in the chromatographic mobility of occupied and unoccupied sites. J. Biol. Chem. 252, 6799-6805.

Skou, J.C. (1965). Enzymatic basis for active transport of Na^+ and K^+ across cell membranes. Physiol. Rev. 45, 596-617.

Smith, D.C.W. (1956). The role of the endocrine organs in the salinity tolerance of the trout. Mem. Soc. Endocrinol. 5, 83-98.

Smith, T.J., and Edelman, I.S. (1979). The role of sodium transport in thyroid thermogenesis. Fed. Proc. 38, 2150-2153.

Snedecor, G.W., and Cochrane, W.G. 1972. Statistical Methods 6th ed. Iowa State University Press, Amer. Iowa. 593 pp.

Sower, S.A., and Schreck, C.B. (1982). Steroid and
    thyroid hormones during sexual maturation of coho
    salmon (Oncorhynchus kisutch) in sea water or
    freshwater. Gen. Comp. Endocrinol. 47, 42-53.

Sumner, F.B. (1944). Vision and guanine production
    in fishes. Proc. Nat'l. Acad. Sci. 30, 285-293.

Surks, M.I., Koerner, D., Dillman, W., and Oppenheimer,
    J.H. (1973). Limited capacity binding sites for
    L-triiodothyronine in rat liver nuclei.
    Localization to the chromatin and partial
    characterization of the L-triiodothyronine-chromatin
    complex. J. Biol. Chem. 248, 7066-7072.

Surks, M.I., Koerner, D.H., and Oppenheimer, J.H. (1975).
    In vitro binding of L-triiodothyronine to receptors
    in rat liver nuclei. Kinetics of binding,
    extraction properties, and lack of requirement for
    cytosol proteins. J. Clin. Invest. 55, 50-60.

Surks, M.I., and Oppenheimer, J.H. (1977). Concentration
    of L-thyroxine and L-triiodothyronine specifically
    bound to nuclear receptors in rat liver and kidney.
    Quantitative evidence favoring a major role of T_3
    in thyroid hormone action. J. Clin. Invest. 60,
    555-562.

Taylor, R.E., and Barker, S.B. (1967). Absence of an

    in vitro effect on oxygen consumption and sodium

    or water transport by anuran skin and bladder.

    Gen. Comp. Endocrinol. 9, 129-134.

Thomopoulos, P., Dastugue, B., and Defer, N. (1974).

    In vitro triiodothyronine binding to non-histone

    proteins from rat liver nuclei. Biochem. Biophys.

    Res. Comm. 58, 499-506.

Thrall, G.L., and Yanagihara, T. (1982). Alterations of

    nuclear thyroid hormone receptors in cerebral

    cortex in vivo. J. Neurochem. 38, 669-674.

Torresani, J., and Anselmet, A. (1978). Partial

    purification and characterization of nuclear

    triiodothyronine binding proteins. Biochem.

    Biophys. Res. Comm. 81, 147-153.

Torresani, J., Anselmet, A., and Wahl, R. (1978).

    Properties of solubilized nuclear triiodothyronine

    binding proteins. Molec. Cell. Endocrinol. 9, 321-323.

Torresani, J., and DeGroot, L.J. (1975). Triiodothyronine

    binding to liver nuclear solubilized proteins in

    vitro. Endocrinology 96, 1201-1209.

Toth, E., and Tabachnick, M. (1979). In vivo demonstration

    of the presence of specific saturable binding sites

    for triiodothyronine in nuclei of tadpole liver.

    Gen. Comp. Endocrinol. 38, 345-355.

Toth, E., and Tabachnick, M. (1980). Effect of temperature on the transport of triiodothyronine (T_3) into liver nuclei of Rana catesbiana tadpoles in vivo. Gen. Comp. Endocrinol. 42, 57-62.

Tsai, J.S., and Samuels, H.H. (1974). Thyroid hormone action: demonstration of putative nuclear receptors in human lymphocytes. J. Clin. Endocrinol. Metabol. 38, 919-922.

Van der Kraak, G.J., and Eales, J.G. (1980). Saturable 3,5,3'-triiodo-L-thyronine-binding sites in liver nuclei of rainbow trout (Salmo gairdneri Richardson). Gen. Comp. Endocrinol. 42, 437-448.

Weirich, R.T., and McNabb, F.M.A. (1981). Nuclear binding sites for triiodothyronine in quail liver. Amer. Zool. 21, 1026.

Widnell, C.C., and Tata, J.R. (1964). A proceedure for the isolation of enzymically active rat-liver nuclei. Biochem. J. 92, 313-317.

Wiersinga, W.M., Chopra, I.J., and Solomon, D.H. (1982). Specific nuclear binding sites of triiodothyronine and reverse triiodothyronine in rat and pork liver: similarities and descrepancies. Endocrinology 110, 2052-2058.

Yoshizato, K., and Nakajima, Y. (1977). Binding characteristics of L-triiodothyronine to isolated rat liver chromatin. Molec. Cell. Endocrinol. 7, 89-100.

Yoshizato, K., Kistler, A., and Frieden. (1975). Binding
of thyroid hormones by nuclei of cells from bull
frog tadpole tail fins. Endocrinology 97, 1030-1035.

Zaugg, H.S., Adams, B.L., and McLain, L.R. (1972).
Steelhead migration: potential temperature effects
as indicated by gill adenosine triphosphatase activities.
Science 176, 415-416.

Zaugg, W.S., and McLain, L.R. (1970). Adenosinetriphosphatase
activity in gills of salmonids: seasonal variations
and salt water influence in coho salmon (Oncorhynchus
kisutch). Comp. Biochem. Physiol. 35, 587-596.

Zaugg, W.S., and McLain, L.R. (1971). Gill sampling as a
method of following biochemical changes: ATPase
activities altered by oubain injection and saltwater
adaptation. Comp. Biochem. Physiol. 38B, 501-506.

Zaugg, W.S., and McLain, L.R. (1972). Changes in gill
adenosinetriphosphatase activity associated with
parr-smolt transformation in steelhead trout, coho,
and spring chinook salmon. J. Fish. Res. Bd. Can.
29, 167-171.

Zaugg, W.S., and McLain, L.R. (1976). Influence of water
temperature on gill sodium, potassium-stimulated
ATPase activity in juvenile coho salmon (Oncorhynchus
kisutch). Comp. Biochem. Physiol. 54A, 419-421.

Zaugg, W.S., and Wagner, H.H. (1973). Gill ATPase activity
    related to parr-smolt transformation and migration
    in steelhead trout (Salmo gairdneri): influence
    of photoperiod and temperature. Comp. Biochem.
    Physiol. 45B, 955-965.

Ziegler-Gunder, I. (1956a). Pterine: Pigmente und
    Wirkstoffe im Tierreich. Biol. Rev. 31, 313-348.

Ziegler-Gunder, I. (1956b). Untersuchungen uber die
    purin-und pterin pigmente in der Haut und in den Augen
    Weissfishe. Z. Vergleiche Physiol. 39, 163-189.

APPENDIX 1:   Probit analysis of T_3-RIA data.

In the T_3-RIA standard curve (A) radioactivity (cpm) in the T_3-antibody fraction of columns exposed to a known amount of carrier T_3 is plotted against the carrier T_3 level.   Plasma sample T_3 concentration can be determined by interpolation.   Sample cpm are compared to a standard curve (A).

Alternatively both standard, and sample, radioactivity may be mathematically transformed to probit or logit values and plotted against the logarithm of standard T_3 concentration (B).   The TCR' was derived by subtracting iodide contamination from the Total Counts Reference (TCR) cpm, representing the total number of counts of $[^{125}I]T_3$ applied to a column. Standard or sample radioactivity (BOUND) was subtracted from the TCR' to calculate FREE (F) cpm.   The BOUND to FREE (B/F) ratio and the $B/F/[B/F]_{0ng\%}$ ratio were determined and subtracted from 1 (ie $1- \dfrac{B/F}{[B/F]0ng\%}$) and the natural logarithm of this value is the PROBIT or LOGIT value.   Plasma T_3 concentrations were calculated from the regression equation derived from the linear relationship between PROBIT values and the natural logarithm of the hormone standard concentration.
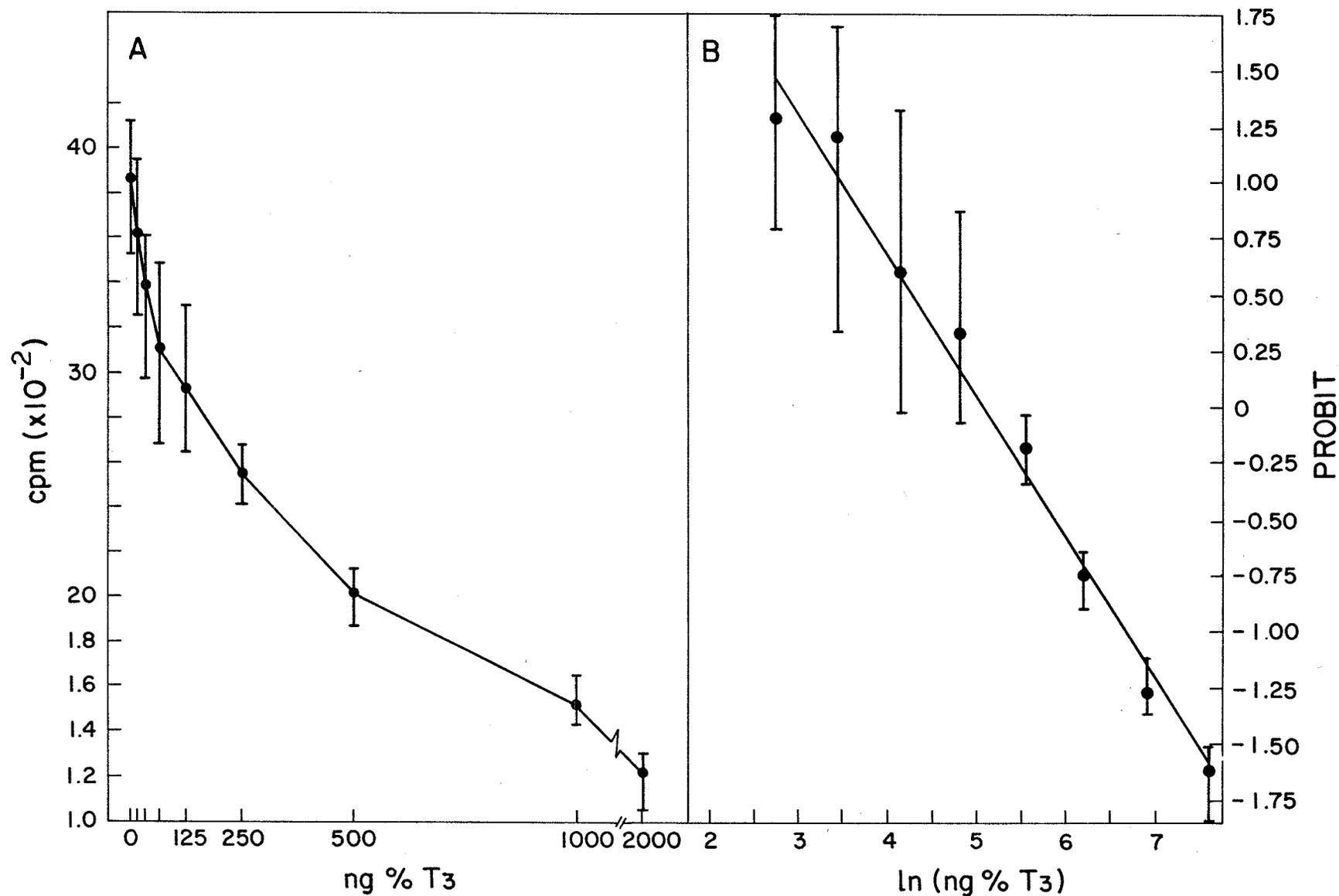
APPENDIX 2:   Model of T_3 interaction with nuclear receptor

and calculation of affinity and capacity of

saturable sites.

Given that T_3R is the T_3 concentration in the

cytoplasm, T_3N is the quantity of T_3 bound to saturable sites

and N is the concentration of unoccupied sites, then

$$T_3R + N \rightleftharpoons T_3N. \tag{1}$$

By definition, k, the equilibrium constant is

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

and, by observing the law of conservation of mass

$$\text{Total liver } T_3 = T_3R + T_3N \tag{3a}$$

$$\text{Total receptor} = M = N + T_3N \tag{3b}$$

and substituting equation (2) with rearrangement of terms

then

$$\frac{T_3R}{T_3N} = T_3R \times \frac{1}{M} + \frac{1}{kM} \tag{4}$$

When $\frac{T_3R}{T_3N}$, $= \frac{F}{B}$ as used in this study, is plotted as a function

of T_3R (F) then, from the linear regression binding capacity

MBC = slope $^{-1}$, and the equilibrium constant k = slope ÷

ordinate intercept.

The Wilkinson inversion plot of enzymology is

reflected in equation (4) (Rodbard, 1973).

APPENDIX 3:   Influence of immersion of trout in T_3, and
freezing of resuspended gill microsomes, on
gill Na^+/K^+-ATPase activity (μmole Pi/hr/mg
protein).

Tissue		Ambient T_3 (μg/100 ml)			
		0	2	5	10
Frozen microsomes	n	–	10	10	4
	$\overline{x}$	–	0.160	0.177	0.161
	SEM	–	0.008	0.018	0.020
Fresh microsomes	n	12	2	2	–
	$\overline{x}$	2.586	4.611	3.716	–
	SEM	0.252	1.659	1.103	–

Fish were immersed in various concentrations of T_3 for
5 days then netted, anaesthetized, bled, and killed by
concussion. Gill microsomes were obtained and frozen in
$N_{2(\ell)}$ then thawed, or, obtained and used fresh. Samples
were then assayed for Na^+/K^+-ATPase activity.

APPENDIX 4. Weights and plasma T_3 concentrations of fish[1] immersed in various concentrations (μg/100 ml) of T_3 for various durations.

HR \ T_3 concn.	Weight (g)				Plasma T_3 (ng/ml)			
	0	2	5	10	0	2	5	10
1	235 (19.6)	175 (7.1)	225 (21.3)	214 (8.9)	0.53 (0.08)	0.48 (0.13)	0.91 (0.24)	1.34 (0.08)
4	216 (11.0)	244 (9.8)	266 (24.3)	205 (23.5)	0.44 (0.06)	0.71 (0.23)	1.21 (0.13)	1.86 (0.28)
8	261 (24.9)	224 (30.8)	252 (33.5)	235 (22.9)	2.04 (0.29)	3.39 (0.83)	5.99 (0.92)	8.99 (0.88)
24	226 (20.4)	229 (27.3)	198 (19.0)	252 (51.5)	1.86 (0.47)	3.59 (0.11)	9.36 (1.37)	12.46 (0.83)
48	219 (12.5)	208 (30.0)	238 (36.8)	277 (23.1)	1.69 (0.36)	3.35 (0.53)	5.68 (1.16)	11.37 (3.06)
96	237 (17.4)	228 (27.5)	254 (28.7)	242 (16.4)	2.85 (0.63)	8.48 (1.62)	3.69 (0.54)	10.49 (0.86)
120	230 (26.2)	238 (30.8)	202 (21.3)	253 (22.6)	1.15 (0.17)	4.01 (0.48)	4.37 (0.86)	11.98 (1.51)
240	202 (17.1)	251 (37.3)	215 (13.6)	194 (16.4)	1.71 (0.56)	3.27 (0.45)	4.63 (0.68)	12.24 (1.00)

[1] $\bar{x}$, (SEM); n = 4.

APPENDIX 5. Plasma T_3 as a function of ambient T_3.

Study	Ambient Level (µg/100 ml)	(pmole/ml)	n	Body Weight[1] (g)	Plasma T_3[1] (pmole/ml)
I	1.25	18.6	9	102.69 (2.74)	16.3 (0.99)
	2.5	37.7	8	103.56 (2.82)	45.3 (2.4)
	5	74.2	8	98.29 (2.84)	131 (10.6)
	10	149	7	95.92 (1.91)	105 (5.9)
	25	371	6	106.87 (3.47)	214 (27.4)
	50	743	6	105.52 (6.78)	690 (37.8)
	100	1486	5	98.98 (4.62)	1099 (50.5)
II	0.125	1.86	5	99.40 (1.88)	0.205 (0.05)
	0.25	3.71	10	97.49 (2.21)	1.39 (0.44)
	0.75	11.1	9	101.15 (3.26)	1.88 (0.37)
	1.00	14.9	6	104.39 (5.79)	3.81 (1.15)
	2.00	29.7	8	107.36 (5.08)	5.16 (1.49)
	10	149	8	101.51 (4.95)	43.3 (10.3)
	50	743	9	104.60 (4.27)	386 (62.1)

[1] $\bar{x}$, (SEM).

APPENDIX 6. Influence of ambient T_3 on trout liver nuclear T_3-binding[1].

Parameter	Ambient T_3 (μg/100 ml)							
	0	0.125	0.25	0.75	1	2	10	50
B/F	43.08 (4.31)	40.08 (1.14)	38.13 (1.78)	23.48 (1.99)	18.22 (1.07)	17.49 (1.99)	10.96 (0.35)	10.43 (0.54)
B/F saturable ($\times 10^3$)	32.39 (4.31)	29.39 (1.14)	27.44 (1.78)	12.78 (1.99)	7.53 (1.07)	6.79 (1.99)		
F/B saturable	32.87 (3.03)	34.28 (1.33)	37.13 (2.12)	90.61 (17.37)	146.2 (19.35)	287.0 (107.4)		
Total Nuclear T_3 (pmole/kg liver)	20.29 (5.07)	54.91 (3.36)	218 (11.4)	255 (16.2)	390 (29.3)	603 (54.3)		
Saturable Nuclear T_3 (pmole/kg liver)	16.00 (4.32)	41.88 (2.77)	162.44 (9.67)	139.3 (15.87)	157.2 (26.23)	205.0 (46.03)		
Free T_3 (nmole/kg)	0.47 (0.11)	1.37 (0.08)	5.75 (0.34)	11.22 (1.00)	28.96 (6.94)	37.55 (6.22)		
Plasma T_3 (pmole/ml)	nil	0.205 (0.050)	1.39 (0.443)	1.88 (0.373)	3.81 (1.15)	5.16 (1.49)	43.3 (10.3)	3.86 (62.1)
n =	10	5	10	9	6	8	8	9

[1] $\bar{x}$, (SEM); n = 6.

APPENDIX 7. Influence of immersion of trout (n=10) in
T_3 (0, 1.25, or 10 μg/100 ml) for 7 days on
plasma T_3 and gill and liver Na^+/K^+-ATPase
activity.

Ambient T_3 (μg/100ml)	0	1.25	10
Fish wt. (g)	97.46[1] (2.08)	96.63 (2.39)	100.85 (2.73)
Plasma T_3 (pmol/ml)	0.47 (0.12)	8.84 (0.61)	73.5 (6.61)
Gill Na^+/K^+-ATPase (μmol Pi/hr/mg protein)	5.742 (0.228)	4.450 (0.273)	4.610 (0.250)
Liver Na^+/K^+-ATPase (μmol Pi/hr/mg protein)	0.318 (0.033)	0.473 (0.047)	0.366[2] (0.061)

[1] $\bar{x}$, (SEM).

[2] n = 9.

APPENDIX 8. Influence of immersion of trout (n=10) in various concentrations of T_3 for 6 or 20 days in plasma T_3 levels and gill Na^+/K^+-ATPase activity and integumentary guanine content.

Parameter	Days immersed	Ambient T_3 (μg/100 ml)		
		0	2	10
Body weight (g)	6	325.91 (30.33)[1]	278.64 (75.82)	302.95 (29.9)
	20	281.84 (26.94)	264.42 (25.36)	303.97 (22.56)
Plasma T_3 (pmol/ml)	6	4.50 (0.47)	9.67 (0.70)	25.1 (1.24)
	20	5.01 (0.87)	8.43 (0.31)	45.4 (2.28)
Gill Na^+/K^+-ATPase (μmole Pi/hr/mg protein)	6	3.672 (0.102)	2.693 (0.156)	2.287 (0.199)
Integumentary guanine (μmole/cm^2)	20	0.693 (0.045)	0.678 (0.050)	0.847 (0.069)

[1] $\bar{x}$, (SEM).