INVESTIGATIONS INTO THE PATHOGENESIS AND MANAGEMENT OF HYPERTHYROIDISM AND THYROID HORMONE DEIODINATION IN THE DOMESTIC CAT

BY

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DECLARATION OF ORIGINALITY

I declare that the composition of this thesis and the work presented herein is my own. Work performed by others as part of collaborative studies are indicated in the acknowledgement section.

Darren James Foster

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ABSTRACT

Hyperthyroidism caused by toxic nodular goitre, is the commonest endocrine disease of the domestic cat. Despite this, little is known about feline thyroid physiology, pathophysiology, nor the pathogenesis of the disease. Since L-triiodothyronine (T3) is the hormone responsible for the major clinical manifestations of thyrotoxicosis, investigations are here reported into the hepatic, renal and thyroidal expression of type 1 iodothyronine deiodinase (IDI), the selenoenzyme responsible for conversion of L-thyroxine (T4) to T3 in these tissues. Studies were carried out into the possible role of thyroid stimulating immunoglobulins, selenium status and somatic mutations of the thyrotropin receptor (TSHR) in the pathogenesis of the disease. Finally, data are presented on a previously unreported method of presurgical management of the disease using a combination of potassium iodate and propranolol.

While feline liver and kidney were found to express IDI at similar concentrations, and the feline enzyme is able to metabolise T4 at a similar rate to that of rats, feline IDI is unusual in that it has little ability to metabolise reverse triiodothyronine which is the preferred substrate for this enzyme in rats and humans. Additionally, unlike all other carnivores and omnivores studied thus far, cats were not found to express thyroidal IDI.

Using feline thyrocytes, Chinese hamster ovary cells expressing human TSHR (JPO9) and the rat thyrocyte cell line FRTL-5, no populations of immunoglobulins were detectable in the sera of hyperthyroid cats which stimulated cAMP production, displaced TSH binding from its receptor or induced growth compared to euthyroid cats.

The plasma selenium status of cats from areas with high (Edinburgh and Sydney) and low (Denmark and Perth) incidences, respectively, of hyperthyroidism was not significantly different from each other. Cats however, have plasma selenium concentrations and red blood cell glutathione peroxidase activities which are approximately 10 times that of selenium replete rats and humans.

In 11 hyperthyroid cats, the DNA for the TSHR region between codons 480 and 640 (the most common site for somatic mutations in human toxic nodular goitre) was not found to contain any such mutations.

The mainstay of presurgical treatment for hyperthyroidism is the use of carbimazole, a drug which is not well-tolerated by approximately 8 per cent of affected cats. A combination of propranolol and potassium iodate was found to normalise heart rates, serum T3, and to a lesser extent T4 concentrations in a significant number of cats and may be used as an alternative presurgical treatment in those cats which cannot tolerate carbimazole.

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ABBREVIATIONS

A23187	calcium ionophore, A23187
ALT	alanine aminotransferase
ANA	antinuclear antibodies
ANOVA	analysis of variance
AST	aspartate aminotransferase
ATP	adenosine triphosphate
AuG	aurothioglucose
BP	base pair
^{125}IBrAcrT3	^{125}I-bromoacetyl-rT3 affinity label
BSA	bovine serum albumin
bTSH	bovine thyroid stimulating hormone (thyrotropin)
cAMP	cyclic adenosine monophosphate
CBZ	carbimazole
CHO	Chinese hamster ovary cells
cGMP	cyclic guanine monophosphate
cpm	counts per minute
CyGPX	cytoplasmic glutathione peroxidase
DAG	diacylglycerol
DEAE	diethylaminoethyl
DIT	diiodotyrosine
DLH	domestic long-haired cat
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dpm	degradations per minute
DSH	domestic short-haired cat
DTT	dithiothreitol
EBSS	Earle's balanced salt soution
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EG	euthyroid goitre
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
E-GPX	extracellular glutathione peroxidase
FGF	fibroblast growth factor
FN	female neutered (ovariohysterectomised)
FNG	feline adenomatous thyrocytes
FRTL-5	transformed rat thyroid carcinoma cell line
FSH	follicle stimulating hormone
FT	normal feline thyrocytes
FT3	free L-3,5,3' triiodothyronine
FT4	free L-thyroxine, L-3,5,3',5' tetraiodothyronine
[^{3}H]TDr	tritiated thymidine
HBSS	Hank's balanced salt solution
HCG	human chorionic gonadotrophin
H_2O_2	hydrogen peroxide
ID	iodothyronine deiodinase
IDI	iodothyronine deiodinase type I
IDII	iodothyronine deiodinase type II
IDIII	iodothyronine deiodinase type III
Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G

IgM	immunoglobulin M
IGF-1	insulin-like growth factor 1
IGF-II	insulin-like growth factor II
IL-1	interleukin 1
IP_3	inositol phosphate
IRD	inner ring deiodination
JPO2	untransfected Chinese hamster ovary cells
JPO9	Chinese hamster ovary cells transfected with recombinant human TSHR
KDa	kilodalton
KI	potassium iodate
Km	Michaelis constant
LATS	long-acting thyroid stimulator
LATS-P	long-acting thyroid stimulator-protector
LH	luteinising hormone
MAP	mitogen activating protein kinase C
MIT	monoiodotyrosine
MN	male neutered (castrated)
NaCl	sodium chloride
NCS	newborn calf serum
NSB	non-specific binding
ORD	outer ring deiodination
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIP	hormone-receptor-phospholipase C cascade
PLC	phospholipase C
PMA	phorbol 12-mystrate 13-acetate
PTK	growth factor-tyrosine kinase
PTU	6-n-propyl-2-thiouracil
RBC	red blood cell
RIA	radioimmunoassay
RNA	ribonucleic acid
rT3	L-3,3',5' triiodothyronine
SAP	serum alkaline phosphatase
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide -gel electrophoresis
SECIS	selenocysteine insertion sequence
SEM	standard error mean
SSCP	single stranded conformational polymorphism
TA_2	3,3' diiodothyroacetic acid
TA_3	3,3' triiodothyroacetic acid
TBAB	thyroid blocking antibodies
TBG	thyroid hormone-binding globulin
TBII	thyrotropin receptor-binding inhibitory immunoglobulin
TCA	trichloroacetic acid
TEMED	NNN'N-tetramethylethylenediamine
TETRAC	acetic acid residue of T4
TGI	thyroid growth immunoglobulin
Tg	thyroglobulin
TGF	transforming growth factor
TNG	toxic nodular goitre
TPO	thyroid peroxidase

TRIAC	acetic acid residue of T3
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
TRH	thyrotropin releasing hormone
TBG	thyroxine binding globulin
TR	thioredoxin reductase
TRABs	thyrotropin receptor antibodies
$TRx\text{-}S_2$	oxidised thioredoxin reductase
$TRx\text{-}(SH)_2$	reduced glutathione
TSH	thyrotropin, thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor
TTR	transthyretin
T2	3,3' diiodothyronine
T3	L-3,5,3' triiodothyronine
TT3	total L-3,5,3' triiodothyronine
T4	L-thyroxine, L-3,5,3',5' teraiodothyronine
TT4	total L-thyroxine, L-3,5,3',5' teraiodothyronine
Vmax	limiting velocity of reaction

1.00 INTRODUCTION

1.01 THE FELINE THYROID GLAND, THYROID HORMONES AND CONTROL OF THYROCYTE GROWTH AND FUNCTION

a) ANATOMY AND FUNCTION OF THE THYROID GLAND IN CATS.

The cat has two separate thyroid lobes, regarded by anatomists as a single gland, located on either side of the trachea each covered by a connective tissue sheath. Like in the dog, it is not usually connected by an isthmus as it is in other species such as humans, equids and bovids (Dyce, Sack, and Wensing, 1987). The thyroid glands receive their blood supply via the cranial thyroid artery which arises from the common carotid artery. A caudal or inferior thyroid artery may also supply the glands but is not present in most cats (Nicholas and Swingle, 1924). There are four parathyroid glands in cats, a pair associated with each thyroid gland (one internal, one external for each gland) which share their blood supply with the thyroid glands.

The basic unit of the thyroid gland is the follicle, surrounded by the basement membrane (Capen, 1996). The follicular lumen is filled with colloid which contains thyroglobulin, a large glycoprotein molecule that is the precursor of all thyroid hormones.

b) GENERAL STRUCTURE OF THYROID HORMONES

The main secretory products of the thyroid gland in rats and humans are L- 3,5,3',5' tetraiodothyronine (L-thyroxine, T4), L-3-5-3'-triiodothyronine (T3), and L-3-3'-5' triiodothyronine (reverse T3, rT3). All three are iodinated derivatives of two tyrosine residues coupled by an ether bond and are known as iodothyronines (Figure 1.01). The phenolic ring is regarded as the outer ring and the tyrosyl ring is regarded as the inner ring. Reverse T3 differs from T3 as it has only a single iodine atom on the inner tyrosyl ring and two iodine atoms on the outer phenolic ring (Chopra, 1996).

c) SYNTHESIS AND SECRETION OF THYROID HORMONES

Little is known regarding the specifics of synthesis, secretion or metabolism of thyroid hormones in the domestic cat. What is presented here is a review of general concepts of these areas from studies involving humans and rats.

The functional unit of the thyroid gland is the follicle which is made up of a cluster of follicles surrounding a colloid filled lumen. Colloid consists mainly of thyroglobulin. This is a high molecular weight protein synthesised by thyrocytes and exported and stored in the follicular lumen. Synthesis of thyroid hormones requires iodination of tyrosyl residues on thyroglobulin followed by coupling of these iodinated derivatives. These reactions take place within the follicular lumen at the surface of the apical membrane.

Iodide is actively taken up by thyroid follicular cells via a sodium/iodide cotransport system (the Na^+/"iodine pump") (Taurog, 1996). This action is stimulated by thyroid stimulating hormone (thyrotropin, TSH) via activation of a cyclic adenosine monophosphate (cAMP) mediated second messenger pathway. Iodide is oxidised to a reactive intermediate by thyroid peroxidase (TPO) before being incorporated onto tyrosine residues of acceptor proteins, mainly thyroglobulin. Iodinated tyrosine residues (monoiodotyrosine [MIT] and diiodotyrosine [DIT]) combine to form iodothyronines: either T4 or T3. This reaction takes place in the follicular lumen. The reaction requires the generation of hydrogen peroxide (H_2O_2) in high concentrations. The production of H_2O_2 is the rate limiting step in thyroid hormone synthesis when iodine supply is adequate. Without H_2O_2 TPO has no activity (Taurog, 1996). The thyrocyte is therefore exposed to large quantities of toxic H_2O_2. Production of H_2O_2 is increased by the calcium /phosphoinositol (PIP) cascade but in some animals, cAMP may also be involved (Taurog, 1996). The selenoenzymes glutathione peroxidase and thioredoxin reductase are important in protecting the thyrocyte against oxidative damage.

Two DIT residues coupled to thyroglobulin form T4, and the coupling of one MIT and one DIT form T3 or the biologically inactive isomer rT3.

Thyroglobulin is then transported back into the follicular cell by pinocytosis to be hydrolysed in lysosomes, releasing T4 and T3 which pass out of the thyrocyte into the blood stream mainly by diffusion. Intrathyroidal production of T3 accounts for approximately 20% of circulating T3 in rats and humans. Most of the iodotyrosines are metabolised in the thyroid by deiodination and iodide can then be re-used in thyroid hormone synthesis. Small amounts of thyroglobulin are also released in to the circulation during this process (Figure 1.02).

d) TRANSPORT OF THYROID HORMONES

In humans, thyroid hormones circulate in plasma largely protein bound (99.95 per cent) in order of affinity, by thyroid hormone-binding globulin (TBG) (the existence of TBG remains in doubt in cats), transthyretin (TTR)(previously known as thyroxine-binding prealbumin) and albumin (Chopra, 1996; Beckett and Wilkinson, 1998; Hard, 1998). Less than 0.05 per cent and 0.1 per cent of T4 and T3 respectively, circulate unbound ('free') in plasma but it is these concentrations which have biological effects and initiate negative feedback mechanisms (Chopra, 1996).

e) BIOLOGICAL ROLE OF THYROID HORMONES

Thyroxine is the most abundant iodothyronine in thyroglobulin being about 10 to 20 times more abundant than T3, and 30 to 100 times more abundant than rT3 (Chopra, 1996). Triiodothyronine is several times more potent, and is less avidly bound in serum, than T4 (Chopra, 1996). The peripheral deiodination of T4 to T3 is discussed in detail in Chapter 1.01 (g,h and i). Reverse T3 has no metabolic activity and is rapidly deiodinated to 3, 3' diiodothyronine (T2).

Besides T4, T3 and rT3, several other iodothyronines are found in sera. These include three diiodothyronines (3,3'-T2, 3,5-T2 and 3',5'-T2), two monoiodothyronines (3'-T1 and 3-T1), two acetic acid residues of T4 (tetrac) and T3 (triac) and the sulphate and glucuronide conjugates of T4, T3 and rT3 (Chopra, 1996). Cats have poor glucuronidation ability.

Thyroxine acts mainly as a prohormone, although there is still some debate regarding possible roles in metabolism and the regulation of the thyroid gland. The importance of T4 and T3 is tissue specific. The brain requires plasma T4 but not T3 for development whilst in the kidney, plasma

T3 and not T4, provides the most important source of T3 acting at nuclear receptors (Beckett and Wilkinson, 1998). Reverse T3 has been shown to have thyroidal agonistic and antagonistic activity in different systems. Other iodothyronines including tetrac and triac also have biological activity, although this is approximately 11 and 21 per cent the calorigenic activity of T4 respectively (Chopra, 1996). However, these two thyroid hormone derivatives are more potent than T4 in other respects. Tetrac is far more potent than T4 in suppressing TSH release from rat pituitary cells (Beckett and Wilkinson, 1998) Tetrac and triac are approximately 10 to 20 times more potent than T4 in stimulating tadpole metamorphosis (Chopra, 1996).

Thyroid hormones enter cells mainly by active transport. Once inside the cell, both T4 and T3 bind to soluble intracellular proteins. The main site of action is the nucleus. Thyroid hormones are necessary for maintaining the metabolism and level of activity of most organs and ensuring development of most tissues in the body including the brain (Uyttersport, Allgeier, Baptist *et al.,* 1997; Mitchell, Nicol, Beckett *et al.,* 1998).

f) GENERAL CONTROL OF THYROID FUNCTION

Thyroid hormone synthesis and secretion are regulated by extrathyroidal (TSH) and intrathyroidal mechanisms (Dumont, Maenhaunt, Pison *et al.,* 1991). TSH secretion is modulated by thyroid hormones via a classical negative feedback mechanism (Uyttersport, Allgeier, Baptist *et al.,* 1997). At the level of the pituitary, 50 per cent of the inhibition of TSH secretion occurs by T3 produced from the local monodeiodination of T4 and 50 per cent from circulating T4 (Table 1.01). The 'thermostat' mechanism of thyroid hormone secretion is the TSH-feedback loop which is modulated by thyrotropin releasing hormone (TRH) from the hypothalamus (Figure 1.03). Release of TRH is under the control of poorly understood mechanisms from higher brain centres (Uyttersport, Allgeier, Baptist *et al.,* 1997).

In most euthyroid animals, the hypothalamic-pituitary-thyroidal axis regulates the control of thyroid hormone production by extrathyroidal (TSH, deiodinases), and intrathyroidal factors (extracellular glutathione peroxidase, E-GPX; hydrogen peroxide, H_2O_2 concentrations). TSH results in increased thyroid hormone synthesis and secretion. Negative feedback on the pituitary, mainly from free T3 (FT3) and free T4 (FT4) reduce TRH secretion. Intrathyroidal regulatory

mechanisms include the Wolff-Chaikoff effect (the inhibition of thyroidal iodine organification by excess iodine intake), decreased TSH receptor sensitivity and alterations in ratios of T4 to T3 production (Nagataki and Yokoyama, 1996).

g) METABOLISM OF THYROID HORMONES-THE IODOTHYRONINE DEIODINASES

The thyroid gland is the sole source of circulating T4 which is regarded largely as a prohormone and is a precursor to all other iodothyronines. In selenium replete humans and rats, approximately 20% of circulating T3 originates from the thyroid gland, whilst 80% of T3 in plasma appears to arise from 5'-mono-deiodination of T4 in a number of tissues especially the liver and kidney (Silva and Larsen, 1986; Kohrle, 1994; St Germain, 1994). T4 has little biological activity and requires 5' monodeiodination to produce the metabolically active hormone T3. Thyroid hormones are metabolised by the sequential removal of iodine atoms from the phenolic (3' or 5' positions) and tyrosine (3 or 5 positions) rings. Deiodination of T4 and other iodothyronines is catalysed by the family of iodothyronine deiodinases (ID) all of which are selenoenzymes. Current evidence suggests that these enzymes provide an autoregulatory role in many tissues to maintain intracellular T3 in response to altered thyroidal secretion of thyroid hormones (St Germain and Croteau 1989; Kohrle, 1994). In addition, the deiodinases appear to be of critical importance during development, ensuring there is a regulated and co-ordinated exposure of specific tissues to thyroid hormones (St Germain and Galton, 1997; Mitchell, Nicol, Beckett *et al.,* 1998; Richard, Hume, Kaptein *et al.,* 1998). Three selenoenzymes are involved in the deiodination of thyroid hormones, namely iodothyronine deiodinase types I, II and III (IDI, IDII, IDIII). IDI is the major isoenzyme found in liver and kidney and under normal circumstances this enzyme appears to provide the major proportion of T3 found in plasma.

The categorisation of these three selenoenzymes was originally derived from their tissue distribution, substrate specificity and sensitivity to propylthiouracil (PTU) and aurothioglucose (AuG). In recent years, each has been cloned and found to have different sequence homologies The characteristics of these three enzymes is presented in Table 1.01. The pathways of thyroid hormone metabolism are illustrated in Figure 1.04. The substrate specificity and kinetic data are illustrated in Table 1.02.

The importance of peripheral deiodination in local hormone control is evident from studies which demonstrate that there are clear tissue differences in the source of T3 which is bound to nuclear T3 receptors (Table 1.03). In the brain, approximately 80% of T3 residing on the nuclear receptors appears to arise from deiodination of T4 within this tissue. By contrast, in the kidney, approximately 90% of nuclear T3 arises from plasma T3 (Silva and Larsen, 1986). Thyroxine may also undergo 5-monodeiodination in many tissues to yield the metabolically inactive molecule rT3. The observation that different tissues have widely different thyroid hormone concentrations appears to suggest that different tissues have different requirements for thyroid hormones (St Germain and Galton, 1997). It has also been speculated that deiodination plays an important role in illness where circulating thyroid hormone concentrations are markedly altered. Deiodination of thyroid hormones thus plays a crucial role in the regulation of T3 supply to both tissue and blood.

Compared to humans and rats, relatively little is known regarding thyroid hormone metabolism in cats. A multicompartmental model investigating T4 and T3 metabolism in the cat demonstrated that the majority of T3 is generated by peripheral metabolism of T4. However, the rate of T3 production appeared to be twice that of the rat and 10 times greater than that reported in humans (Hays, Broome and Turrel, 1988). There is little reported difference in clearance or metabolism of thyroid hormones between euthyroid and hyperthyroid cats (Broome, Hays and Turrel, 1987). In euthyroid and thyrotoxic humans, markedly different thyroid hormone kinetics have been reported (Rosenthal, 1981).

Non-deiodinating pathways of thyroid hormone metabolism include ether bond cleavage, metabolism of the alanine side chain, sulfoconjugation, glucuronidation and O-methylation and account for approximately one third of the metabolism of thyroid hormones (Leonard and Kohrle, 1996).

Table 1.01: Characteristics of the iodothyronine deiodinases in the rat (Types I, II and III).

	Type I	Type II	Type III
Deiodination site	5 and 5'	5'	5
Reaction kinetics	Ping-pong	Sequential	Sequential
Substrate preference	rT3>T4>T3	T4>rT3	T3 (sulphate) >T4
Reaction catalysed	T4 to T3 T3 to T2 rT3 to T2	T4 to T3 rT3 to T2	T4 to rT3 T3 to T2
Tissue distribution	Kidney, liver, thyroid, CNS, pituitary	CNS, BAT, pituitary, thyroid (human)	CNS, placenta, skin
Role	Provides plasma T3	Local T3 production (BAT - plasma T3)	Important roles in foetal development
Propylthiouracil	Inhibits	No effect	No effect
Iopanoic acid	Inhibits	Inhibits	Inhibits
Aurothioglucose	Yes	No	No
Selenoenzyme	Yes	Yes	Yes
Subunit molecular mass (KDa)	27	29	30
Hypothyroidism	Liver, kidney decreased	Increased	Decreased
Hyperthyroidism	Increase	Decrease	Increase
Low T3 syndrome	Decreased	No change	No change
Selenium deficiency	Liver, kidney decreased. Thyroid increased	Decreased	No change
Iodine Deficiency	Liver, kidney no change, thyroid increased	Pituitary no change BAT increased Brain increased	No change

CNS, central nervous system; BAT, brown adipose tissue.

Table 1.02: Kinetic data of IDI deiodination with various substrates

Substrate	Product	Deiodination	K_m*	V_{max}*	V_{max}/K_m
T_4	T_3	phenolic 5'-D	2.3	30	13
T_4S	T_3S	phenolic 5'-D	ND	ND	ND
T_4	r T_3	tyrosyl 5-D	1.9	18	9
T_4S	rT_4S	tyrosyl 5-D	0.3	526	2020
r T_3	3,3'-T_2	phenolic 5'-D	0.06	559	8730
rT_3S	3,3'-T_2S	phenolic 5'-D	0.06	516	8600
T_3	3,3'-T_2	tyrosyl 5-D	6.2	36	6
T_3S	3,3'-T_2S	tyrosyl 5-D	4.6	1050	230
3,3'-T_2	3-T_1	phenolic 3'-D	8.9	188	21
3,3'-T_2S	3-T_1S	phenolic 3'-D	0.3	353	1040

* K_m is expressed in µmol/L and V_{max} in pmol/min/mg of protein.

Adapted from Visser, Vanbuuren, Rutgers *et al.* (1990).

Table 1.03: Origin of T3 bound to nuclear receptors of various tissues (percentage of total nuclear T3)

	Plasma-borne	Locally produced
Pituitary	50	50
Cerebral cortex	20	80
Cerebellum	40	60
Kidney	90	10
Liver	75	25

From Beckett and Arthur (1994)

i) IODOTHYRONINE DEIODINASE - 1 (IDI)

Type I 5' deiodinase is defined by its substrate specificity and the ability of PTU and AuG to inhibit catalysis (Leonard and Kohrle, 1996). In rodents, fish and many higher vertebrates, the highest concentrations of IDI are found in the thyroid followed by the kidney, liver and pituitary and much lower activities in skeletal muscle, heart, spleen, lung, intestine, lactating mammary glands, placenta, salivary glands, brain, white adipose tissue, intestines, skin and nucleated red blood cells (Leonard and Kohrle, 1996). IDI catalyses many reactions such as the outer ring deiodination (ORD) of T4, producing T3, as well as the clearance of plasma T4 by inner ring deiodination (IRD) to produce the inactive metabolite, rT3 (Visser, Kaptein, Terpstra *et al.,* 1988). The site of deiodination by IDI is dependent on the substrate. Thyroxine-sulphate (T4-sulphate) and T3-sulphate undergo preferential tyrosyl ring deiodination, whereas the non-sulphated iodothyronines (T4, rT3) and sulphated 3,3'-diiodothyronine (T2-sulphate) undergo preferential phenolic ring deiodination (St Germain and Galton, 1997).

IDI is expressed in high concentrations in the thyroid of some species such as humans, dogs and rodents but many animal species including goats, cattle and sheep fail to express the enzyme, or at least express it at very low concentrations within the gland (Beckett, Beech, Nicol *et al.,* 1993). In rats and humans, IDI appears to show a substrate preference for rT3, although it can readily act on T4 (Visser, Kaptein, Terpstra *et al.,* 1988).

The current concept of thyroid hormone metabolism is that circulating hormone is transferred to the interstitial space, followed by uptake into cells mainly by active transport. The hormone/ID complex is transported to nuclear receptors where it exerts it thimometic action. Although the subcellular location of IDI is likely to differ from organ to organ, the enzyme is an integral part of the cellular membrane. The localisation of the enzyme to a particular subcellular organelle is largely operational (Leonard and Kohrle, 1996). IDI is often associated with the endoplasmic reticulum in rat liver, whereas in the rat kidney, a plasma membrane localisation of IDI has been reported (Leonard and Kohrle, 1996).

Thyroidal IDI activity increases in selenium depletion unlike hepatic IDI activity which is decreased to approximately 5% of its original value in selenium replete rats (Beckett, Beddows,

Morrice *et al.,* 1987; Beckett, Russel, Nicol *et al.,* 1992; Beckett, Beech, Nicol *et al.,* 1993; Beech, Walker, Beckett *et al.,* 1995).

A number of methods have been employed to assay for IDI expression and activity. Expression of the enzyme is usually assessed with an affinity labelling technique using ^{125}I-bromoacetyl derivatives of rT3, T3 or T4 (Kohrle, Rasmussen, Ekenbarger *et al.,* 1990; Schoenmakers, Pigmans and Visser, 1992). Activity is measured by assessing the ability of IDI to metabolise T4 or rT3 in the presence of an active thiol agent such as dithiothreitol (DTT) (Leonard and Rosenberg, 1978; Visser, and Overmeeren-Kaptein, 1981; Moreno, Berry, Horst *et al.,* 1994). IDI and IDII show very different sensitivities to inhibition by the addition of PTU and AuG. IDI activity with rT3 or T4 as substrate is readily inhibited by both these agents, whilst IDII is relatively resistant to inhibition by PTU or AuG (St Germain, 1994). These inhibitors are thus often used to indicate which isoenzyme is expressed by tissues (Table 1.02).

Previous studies have suggested that IDI has similar properties in all species investigated. The main differences reported have been confined to small variations in molecular mass (Schoenmakers, Pigmans and Visser, 1992), differences in turnover number of approximately 10-fold between species (Santini, Chopra, Hurd *et al.,* 1992; Schoenmakers, Pigmans and Visser, 1992) and differences in sensitivity to inhibition by AuG (Santini, Chopra, Hurd *et al.,* 1992). However there have been reported clear species differences concerning the degree of IDI expression by the thyroid (Beckett, Beech, Nicol *et al.,* 1993; Beech, Walker, Beckett *et al.*1993).

Kinetic reactions of IDI within and between different species have yielded widely different results. Some of the reasons for this include failure to measure deiodination under optimal conditions (substrate, time and pH dependent factors), the use of different co-factors and at different concentrations, the failure to establish the stability of reaction products and the lack of appropriate conditions to examine a multisubstrate reaction (Leonard and Kohrle, 1996). However, despite these variations between laboratories, investigations into differences between deiodination by different species within laboratories are still valid.

As the removal of iodine atoms is accompanied by the replacement with hydrogen atoms (a reduction reaction), cofactors are necessary to facilitate this transfer. Early studies demonstrated

that removal of the cytosolic component from an homogenate decreases IDI activity. The return of the cytosolic fraction to the microsomal fraction, or addition of NADPH, reduced glutathione, reduced monothiols or dithiols (such as dithiothreitol) restore activity (Leonard and Kohrle, 1996).

The deiodination reaction mechanism is referred to as a classic "ping-pong" reaction, where the first half of the reaction involves the removal of an iodine atom and the subsequent formation of an oxidised enzyme intermediate. The second half of the reaction then involves reduction of the second substrate by a reduced thiol co-factor enabling the enzyme to enter another deiodinating cycle (Leonard and Kohrle, 1996). The inhibition of this reaction by PTU and AuG involves the formation of a catalytically inactive, mixed selenosulfide (Leonard and Kohrle, 1996)(Figure 1.05). Thiouracils are competitive inhibitors with respect to the cofactors and non-competitive inhibitors with respect to the substrate (iodothyronines) (Leonard and Kohrle, 1996). A decrease in concentration of cofactor or increase in concentration of thiouracils results in reduction of Km (Michaelis constant) and Vmax (the limiting velocity of the reaction) (Leonard and Kohrle, 1996).

Prior to cloning, it seemed that a third 5' deiodinase enzyme existed. It appeared that the liver and kidney expressed two types of enzymes depending on the concentration of cofactor. When DTT was used at 20mM, a high Km enzyme (values in the micromolar range) was observed. When glutathione, or a reconstituted thioredoxin system were used as cofactors, a low Km enzyme (values in the nanamolar range) was observed (St Germain and Galton, 1997). However, it was subsequently demonstrated that the rat G21 IDI cDNA encoded for both types of enzymes depending on the cofactor used (St Germain and Galton, 1997). Therefore, there is only one IDI enzyme with kinetics that vary considerably, depending on the cofactor used.

The cDNA and mRNA for IDI in humans, dogs, mice, chicks and tilapia have been cloned illustrating several important features (St Germain and Galton, 1997). The cDNA for IDI encodes a selenocysteine insertion sequence (SECIS) in the 3' untranslated region of the mRNA (Berry, Banu, Chen *et al.,* 1991). There are two essential histidines at amino acid residues 158 and 174 (Berry, 1992). The phenylalanine at residue 65 of the human IDI is of critical importance in the ability of the enzyme to catalyse the 5' deiodination of rT3 and T2-sulphate (Toyoda, Harney, Berry *et al.,* 1994). The cDNA sequence for IDI is highly conserved except for dog IDI which has a leucine for phenylalanine substitution at amino acid 65 making it inefficient in catalysing 5'

deiodination of rT3 and T2 sulphate (St Germain and Galton, 1997). The IDI of tilapia is relatively resistant to inhibition by PTU and AuG although the structural characteristics responsible for this difference are unknown (St Germain and Galton, 1997). The selenocysteine residue is critical for the catabolism of thyroid hormones as site-directed mutagenesis studies confirm by replacing the selenocysteine residue with a cysteine residue (Berry, Kieffer, Harney *et al.,* 1993).

Sulphation of T3 and 3,3'-T2, but not rT3, markedly enhances the catabolism of these iodothyronines (Visser, Kaptein, Terpstra *et al.,* 1988; Visser, Vanbuuren, Rutgers *et al.,* 1990; Rutgers, Heusdens and Visser, 1991). Substitution of the alanine side-chain of T3 and 3,3'-T2 with acetic acid resulting in 3,3',5-triiodothyroacetic acid (TA_3) and 3,3'-diiodothyroacetic acid (3,3'-TA_2) enhances deiodination 16- and 3-fold respectively (Rutgers, Heusdens and Visser, 1991). The IRD of TA_3 and the ORD of 3,3'- TA_2 are further stimulated about 50 times by 4' sulphation. In fact, TA_3-sulphate is the preferred substrate for IDI (Rutgers, Heusdens and Visser, 1991).

ii) IODOTHYRONINE DEIODINASE TYPE 2 (IDII)

IDII is not expressed by liver or kidney, but the enzyme appears to provide an important intracellular source of T3 in many other tissues including the central nervous system, pituitary, thyroid and brown adipose tissue of some species including the rat. IDII catalyses only 5'-monodeiodination, has T4 as its preferred substrate over rT3 and has a sequential mechanism of reaction, as opposed to the ping-pong mechanism of IDI. Like IDI, IDII has an absolute *in vitro* requirement for reduced thiols (Leonard and Kohrle, 1996). The physiological cofactor has yet to be determined. Like IDI, IDII has a highly conserved TGA selenocysteine codon but for reasons which are unknown, it is not inhibited by AuG or PTU.

iii) IODOTHYRONINE DEIODINASE TYPE 3 (IDIII)

IDIII performs only 5-monodeiodination, acts on T3 in preference to T4 and also has an absolute *in vitro* requirement for reduced thiols. High concentrations are found in the central nervous system, placenta and skin. IDIII shares 70% sequence homology with IDI and contains a highly conserved TGA selenocysteine codon at the active site (Leonard and Kohrle, 1996). In recent

years, IDIII has been shown to have an important role in the development of the neonate. IDIII activity is highest in the newborn especially in the brain where it serves to protect the neural tissue from T3 (St Germain and Galton, 1997). IDIII is also highly expressed in the placenta, again acting as a barrier to protect the foetus against T4 and T3 (St Germain and Galton, 1997).

1.02 CONTROL OF GROWTH AND FUNCTION OF THYROCYTES IN HEALTH

a) THE THYROTROPIN RECEPTOR AND CELL SIGNALLING WITHIN THE THYROID GLAND

i) Structure of the TSH receptor

Generally, three major types of receptor exist: channel linked, catalytic and G-protein linked. The TSH receptor belongs to the large family of G-protein linked receptors. These group of receptors consist of seven hydrophobic transmembrane segments, three extracellular loops, three intracellular loops, an extracellular amino terminus and an intracellular carboxyl terminus (Paschke, Parmentier and Vassart, 1994; Uyttersport, Allgeier, Baptist *et al.,* 1997). TSH, luteinising hormone (LH) and follicle stimulating hormone (FSH) are coupled mainly to the generation of cAMP through membrane-bound adenylate cyclase (Paschke, Parmentier and Vassart, 1994). It is estimated to have a non-glycosylated Mr of 82KDa (Ludgate, and Vassart, 1995).

The TSH receptor was first cloned in 1989 (Parmentier, Libert, Maenhaunt, 1989). Although its sequence is generally quite homologous, TSH receptors vary morphologically between species. The TSH receptor is encoded by 10 exons which spread over 60 kb on chromosome 14. The large extracellular domain is encoded by nine exons, whereas the intracellular and extracellular loops, the transmembrane segments and the C terminus are encoded by exon 10 (Paschke, Parmentier and Vassart, 1994). The extracellular domain is important for the binding of TSH (Ludgate and Vassart, 1995) and therefore subsequently the generation of cAMP (Paschke, Tonacchera, Van Sande *et al.,* 1994) (Figure 1.06). The TSH receptor is characterised by a very long intracellular N terminal domain and a short third intracellular loop and is very homologous to both LH and FSH receptors (Uyttersport, Allgeier, Baptist *et al.,* 1997).

ii) G protein signalling

The TSH receptor is one of almost 2000 G protein-coupled receptors that have been reported. They are divided into approximately 100 families which are classified according to sequence homology, ligand structure and receptor function. Significant G protein sequence homology occurs within families, but between families there is little or no homology. (Ji, Grossman and Ji, 1998).

G proteins are trimers whose function [to transmit signals from transmembrane receptors to intracellular enzymes and ion channels (Iiri, Farfel and Bourne, 1998)], depends upon the ability of the protein to dissociate into an α monomer and $\beta\gamma$ dimer. The dissociation is triggered by the activation of an associated receptor (Figure 1.07).

All G proteins have seven transmembrane segments with the amino terminus in the extracellular space, three extracellular loops, three intracytoplasmic loops and a cytoplasmic carboxyl terminus. Regardless of the subclass of G-protein they commonly promote exchange of GDP for GTP on the α subunit. The GTP bound α subunit then activates adenylate cyclase. The G_s protein eventually acts as a GTPase converting GTP to GDP, which terminates the adenylate activity. A receptor may be coupled to one or more G proteins (Vassart, Desarnaud, Duprez *et al.,* 1995).

G proteins are classified into many different classes for example, G_s, G_i, G_q and G_{12}. The TSH receptor mainly binds to G_s (leading to cAMP accumulation) although at high concentrations of TSH, will bind G_q activating the inositolphosphate diacylglycerol cascade (Laurent, Mockel, Van Sande *et al.,* 1987; Vassart, Desarnaud, Duprez *et al.,* 1995; Dhanasekaran, 1998; Dhanasekaran, Tsim, Dermott *et al.,* 1998). The classes of some G proteins, their effectors and actions are listed in Table 1.04.

Table 1.04: Classification of G-proteins, the action of their effectors and second messenger activation.

Class	Effector function	Second messenger
s	Stimulates adenyl cyclase	↑ cAMP
i	Inhibits adenylate cyclase Opens K^+ channels	↓ cAMP ↑ membrane potential
o	Closes Ca^{++} channels	↓ membrane potential
q	Activates phospholipase c	↑ IP_3, DAG
t	Stimulates cGMP	↓ cGMP

cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanine monophosphate; DAG, diacylglycerol; IP_3, inositol phosphate

iii) Second messenger systems in the thyrocyte

Second messenger systems transform extracellular signals into intracellular ones. The importance of each second messenger pathway within the thyroid gland is dependent on the species involved and the affector being studied. Exposure to, and reactions with, cytokines may also alter the response of second messenger pathways.

In the thyroid gland, at least three distinct second messenger pathways have been well defined: (1) the hormone receptor-adenylate cyclase-cAMP protein kinase system, (2) the hormone receptor-tyrosine protein kinase pathway and (3) the hormone-receptor-phospholipase C cascade (PIP) (Dumont, Takeuchi, Lamy *et al.,* 1981; Dhanasekaran, 1998) (Figure 1.08).

In all species studied to date (dog, rat, mouse, sheep, pig, bovine, horse and human), TSH and forskolin (a diterpene compound that activates adenylate cyclase) induce cAMP accumulation through stimulation of adenylate cyclase via G protein activation (Raspe, Reuse, Maenhaut *et al.,* 1989). Although TSH increases cAMP accumulation in many species, its effects on thyroid function, differentiation and proliferation are species-specific. In dog thyrocytes and FRTL-5 cells, TSH at physiological concentrations will stimulate only the cAMP cascade. At three times these concentrations, TSH will also activate the PIP cascade through G_q binding (Vassart and Dumont, 1992). In human thyrocytes, TSH activates the cAMP cascade and the PIP cascade at 10 times the concentration of TSH required for cAMP activation alone (Vassart and Dumont, 1992; Ludgate, and Vassart, 1995; Uyttersport, Allgeier, Baptist *et al.,* 1997; Hard, 1998). The functions of TSH in the control of thyrocyte function in different species are therefore species specific. In thyrocytes where only the cAMP cascade is activated, TSH effects are mediated only through cAMP, which enhances functional characteristics of thyrocytes including iodide transport, thyroglobulin synthesis, iodide oxidation and thyroid hormone synthesis within thyroglobulin, and the endocytosis and digestion of thyroglobulin with release of thyroid hormones (Raspe, Reuse, Maenhaut *et al.,* 1989; Corvilain, Laurent, Lecomte *et al.,* 1994). In the human thyroid where TSH activates both cAMP and PIP, the latter cascade also stimulates iodination and thyroid hormone synthesis (Vassart and Dumont, 1992; Hard, 1998).

The non-physiological concentrations of TSH required to stimulate phospholipase activation have cast doubt over the relevance of this pathway in TSH action. However, it has been shown that after cAMP activation occurs, the TSH receptor down regulates its cAMP response and phospholipase activity is increased. In addition, fibroblast growth factor (FGF) causes downregulation of TSH induced cAMP accumulation (Burrow and Eggo, 1994). There is therefore, significant cross talk between the tyrosine kinase, PIP and cAMP pathways in thyrocytes (Burrow and Eggo, 1994; Hard, 1998). In the dog thyrocyte, TSH has not been demonstrated to activate the PIP cascade, although it activates all classes of G proteins (Uyttersport, Allgeier, Baptist *et al.,* 1997; Hard, 1998). There is no present explanation for the discrepancy between activation of G_q and lack of PLC activity.

TSH leads to a marked increase in protein deiodination in the dog, mouse, and bovine but this effect is weak in the rat, pig and horse (Raspe, Reuse, Maenhaut *et al.,* 1989). In all species, TSH increases thyroid hormone secretion through stimulation of adenylate cyclase and not through the Ca^{++} - PIP cascade. However, not all effects of TSH are mediated through cAMP which may be the consequence of different populations of TSH receptors or effectors (Uyttersport, Allgeier, Baptist *et al.,* 1997), but is more likely to be the result of different species affinity for G-proteins that are associated with the TSH receptor (Vassart and Dumont, 1992). Like other receptors, the TSH receptor down-regulates its response following stimulation by thyrotropin. Because of the ubiquitous nature of steroid receptors such as the TSH receptor, and the close similarity of the sequence of steroid hormones, activation of steroid receptors is sometimes non-specific. For example, stimulation of the TSH receptor can occur with TSH, human chorionic gonadotrophin (HCG) and LH (Yoshimura, Hershman, Pang *et al.,* 1993).

The thyrocyte is also regulated by several neurotransmitters (noradrenaline through α and β receptors, acetylcholine through muscurinic receptors) that modulate adenylate cyclase and or phospholipase C through their receptors (Dumont, Takeuchi, Lamy *et al.,* 1981). The roles of neurotransmitters vary between species and a physiological role has yet to be determined (Uyttersport, Allgeier, Baptist *et al.,* 1997).

b) CONTROL OF THYROCYTE GROWTH

The development of primary cultures from a number of species has lead to the understanding that a number of factors influence the growth of thyrocytes (Burrow and Eggo, 1994). Cell growth can be mediated by receptors that belong to families such as G-protein coupled receptors, receptor tyrosine kinases, cytokine receptors, cell adhesion receptors and antigen receptors (Dhanasekaran, 1998).

Proliferation of thyroid follicular cells is controlled by many intracellular cascades with significant cross talk as described previously. These include cAMP, phorbol-ester-PKC, inositol 1,4,5-triphosphate (IP_3)/Ca^{2+}/diacylglycerol (DAG), the growth-factor-tyrosine kinases (PTK) and mitogen activated protein kinases (MAP kinases) that are activated by a distinct group of extracellular signals and receptors (Ledent, Denef, Cottecchia *et al.,* 1997; Uyttersport, Allgeier, Baptist *et al.,* 1997). The importance and contribution of each of these pathways is highly species-specific. Indeed, these signals may differ within the thyroid gland of an animal depending on the degree of natural heterogeneity of thyroid cells. The cAMP cascade promotes proliferation and differentiation whilst PKC and PTK promote proliferation and loss of differentiation (Uyttersport, Allgeier, Baptist *et al.,* 1997).

Thyroid cells grow mainly in adolescence and then slow down to divide approximately five times during adult life (Dumont, Takeuchi, Lamy *et al.,* 1981) It may be that not all cells divide, and there may be small subpopulations of cells which slowly divide to replace dying cells. Although thyrocytes do not divide greatly in adult life they certainly retain the propensity for growth in times of iodine deficiency (Uyttersport, Allgeier, Baptist *et al.,* 1997) and in the presence of goitrogenic substances (for example thiouracil, sulfonamides and Brassica species of plants) (Dumont, Maenhaut, Pison *et al.,* 1991).

i) TSH-induced growth

It is now generally accepted that TSH is one of the major mitogens for thyrocytes. However, It has not always been possible to demonstrate TSH-induced growth in all species using *in vitro* techniques. Whilst the thyrocytes of some species will grow in response to TSH acting primarily

through the cAMP pathway (Burrow and Eggo, 1994), this effect (at least *in vitro*) is not maintained and begins to slow with time, suggesting that the TSH receptor eventually becomes desensitised to growth but not necessarily other functions (Dumont, Maenhaut, Pison *et al.,* 1991; Hard, 1998). Thyrocytes also go some way to controlling the growth of neighbouring thyrocytes through release of cytokines (Dumont, Maenhaut, Pison *et al.,* 1991). Thyrocytes can grow independently of TSH, for example, in anencephalic animals (Dumont, Maenhaut, Pison *et al.,* 1991). Growth can also be inhibited or enhanced by paracrine or autocrine factors but these factors have species-specific effects (Dumont, Maenhaut, Pison *et al.,* 1991; Brown, 1995). For example, the extracellular matrix (ECM) also exhibits controlling effects on growth and may explain some of the differences in results between laboratories studying growth effects .

TSH is mitogenic for thyrocytes in a number of species (dogs, humans, and rats) and FRTL-5 cells, but not in others (porcine, bovine and ovine) (Ollis, Davies, Munro *et al.,* 1986; Dumont, Maenhaut, Pison *et al.,* 1991). In those species where it acts as a mitogen, it may do so only in the presence of other growth factors such as IGF-1 or insulin. The lack of growth stimulation in response to TSH may be due to inaccessibility of the TSH receptor in *in vitro* models, lack of an essential factor in the culture medium, lack of growth potential in cell preparations or true unresponsiveness to TSH.

ii) EGF-induced growth effects

EGF acts through its own receptor (EGFR) via an independent, tyrosine kinase second messenger system. It is known to influence the growth and function of most mammalian cells (Nilsson, 1995). It is likely that the majority of its actions are mediated in a paracrine or autocrine manner (Nilsson, 1995; Hard, 1998). EGF is mitogenic for human, canine, porcine, ovine, and bovine thyrocytes *in vitro.* In addition, *in vivo* it is mitogenic for thyrocytes in ovids and mice (Dumont, Maenhaut, Pison *et al.,* 1991). It does not produce growth in FRTL-5 cells as some strains lack EGF receptors (Burrow, and Eggo, 1994). EGF also results in poor differentiation and receptor expression and function and inhibits TSH-mediated thyroid functions (Hard, 1998). In many species, differences occur in thyrocyte response to EGF. In porcine, unlike canine thyrocytes, EGF increases intracellular calcium accumulation (Raspe, Reuse, Maenhaut *et al.,*

1989). EGF also leads to induction of certain oncogenes such as c-fos and c-myc (Raspe, Reuse, Maenhaut *et al.,* 1989) which are associated with cell proliferation (Brown, 1995).

iii) IGF-I, IGF-II and FGF

IGF-1 stimulates growth in porcine cells. Both IGF-I and II increase growth in ovine and human thyrocytes, and FRTL-5 cells (Dumont, Maenhaut, Pison *et al.,* 1991). FGF acts through the tyrosine kinase and phospholipase pathways to induce growth. FGFs have been found to be produced by FRTL-5 cells and human thyrocytes (Hard, 1998) although FGF is not mitogenic for human thyrocytes (Burrow, and Eggo, 1994).

It is generally accepted that more than one factor, and more than one intracellular signal, are responsible for the proliferation of thyrocytes (Tramontano and Villone, 1994). Many of these effects occur through G-protein signalling (point mutations in the $G\alpha_s$ (gsp oncogene) (Dhanasekaran, Tsim, Dermott *et al.*, 1998) and Ras gene expression, a universal signalling pathway through which many of the signals that direct cells to divide act (Burrow and Eggo, 1994). In addition, thyrocytes also control their own growth by the release of paracrine factors such as IGF-I and II, FGF, transforming growth factor-β (TGF-ß), glucagon, and IL-6 (Dumont, Maenhaut, Pison *et al.,* 1991).

1.03 THYROID STIMULATING IMMUNOGLOBULINS

a) CATEGORIES OF THYROID STIMULATING IMMUNOGLOBULINS

The best example of thyroid disease induced by an autoantigen is Graves' disease. Autoantigens in Graves' disease are directed against thyroglobulin (Tg), thyroid peroxidase (TPO) and the TSH receptor. The latter is the primary autoantigen involved in the pathophysiology of the condition (Davies, 1996). Long-acting thyroid stimulator (LATS) was first reported in 1956 (Adams and Purves, 1956). Later it was discovered that this stimulus existed in the immunoglobulin (Ig) G (IgG) fraction and would inhibit TSH from binding to its receptor. Since that time, substantial work has allowed the subclassification of these immunoglobulins.

Considerable confusion exists regarding antibodies that bind to the TSH receptor, resulting mainly from the differing nomenclature used by various groups (Ludgate and Vassart, 1995). Antibodies that bind to the TSHR may not initiate an intracellular signal transduction process. The four main types of antibodies are: thyroid stimulating immunoglobulins (TSIs) which stimulate the production of cAMP and are characteristic of Graves' disease, TSH binding inhibitory immunoglobulins (TBIIs) which inhibit the binding of TSH to its receptor, thyroid blocking antibodies (TBAB) which inhibit the accumulation of cAMP and are the probable cause of hypothyroidism in some cases of idiopathic myxoedema (Ludgate and Vassart, 1995) and thyroid growth stimulating immunoglobulins (TGIs). In human Graves' patients, TSIs are sometimes subclassified into LATS or LATS-protector (LATS-P). LATS stimulate thyroid activity in several species whereas LATS-P are thyroid stimulators specific for human thyroid tissue (Smith and Hall, 1974). Graves' IgG is a classic TSI that also has growth promoting activity *in vivo* and *in vitro* depending on the reporter cell used. The role of stimulating immunoglobulins in Graves' disease is well understood, whilst the role of TSIs or TGIs in forms of 'non-immunogenic' thyrotoxicosis, such as toxic nodular goitre, are more controversial.

The difference in functional characteristics of these immunoglobulins in Graves' disease may relate to their molecular binding characteristics allowing activation of specific second messenger pathways (Davies, 1996). It is possible that a number of non-immunogenic forms of

thyrotoxicosis have as part of their pathogenesis and/or pathophysiology, the presence of humoral factors which bind to the TSHR, altering function and/or growth of thyrocytes. These humoral factors may be endocrine, paracrine or apocrine in nature, initiate the disease, or appear after the main pathological process has begun.

b) DIFFICULTIES IN INTERPRETING DATA REGARDING HUMORAL STIMULI FOR GROWTH AND FUNCTION OF THYROCYTES

The difficulties in detecting these humoral stimuli result from several factors which include the type of disease investigated, the population of patients studied and their iodine status, the cell reporter systems used, the methods of purification of immunoglobulins, the experimental conditions and the end point of detection of 'stimulus' (e.g. second messenger, growth stimulation).

What follows is a brief discussion of the problems in scientific method, and evidence for and against the presence of a chronic low grade growth stimulator (presumably IgG) leading to the formation of goitre in humans and felids. Particular attention is paid to growth stimulation.

i) Specificity of cell types

The TSH receptor has low specificity across species and, therefore, different thyroid cell types (both primary cultures and manipulated cell lines) can be used to infer mechanisms of actions in other species (Dumont, Takeuchi, Lamy *et al.,* 1981). Immortal cell lines and, perhaps to a lesser extent primary cell types, have been manipulated and function in an abnormal way when compared to normal thyroid follicles. The other disadvantage of using immortalised cells are that they represent an *in vitro* situation and any mechanism of action must be implied because of the lack of many other possible contributing growth factors. Additionally, most cell systems employ monolayer cells, which are not true representations of the three dimensional structure of normal thyroid tissue (i.e. the follicle).

ii) Purification of immunoglobulins

The method of purification of immunoglobulins is important for two reasons. Firstly, impurities (such as IL-1, EGF, and IGF-1) may have some inherent thyroid stimulating activity (Zakarija and McKenzie, 1987) resulting in false positive results, and, secondly, the immunoglobulin may be inactivated during preparation causing it to lose its stimulating potential (Brown, Kertiles and Kleinmann, 1986).

According to Brown, Kertiles and Kleinmann (1986), the most desirable methods for IgG purification are anion exchange with diethylaminoethyl (DEAE) cellulose or protein A affinity chromatography, both for their purity of extract and, consequently, inhibition of TSH binding. However, even protein A affinity chromatography results in elution of IgA and IgM in small quantities and cannot be considered 100 per cent specific for IgG. Protein G affinity chromatography may be more specific.

iii) Thyroid stimulating immunoglobulins and cAMP accumulation as a marker

TSH receptor antibodies (TRABs) prevent TSH binding to its receptor. Various studies have shown that TRABs positive sera from Graves' patients cause stimulation of adenylate cyclase assayed by cAMP accumulation (Kasagi, Konishi, Iida *et al.,* 1982; Kasagi, Hatabu, Tokuda *et al.,* 1988). A number of factors affect the sensitivity and specificity of these assay systems and are discussed in more detail in Chapter 5. Briefly, the degree of stimulation is dependent on the TRABs titre and whether sera were unpurified, whether immunoglobulins of all classes were isolated (by polyethylene (PEG) or ammonium sulphate precipitation) or pure IgG was extracted. The response is also dependent on the cell system used and on the experimental conditions. Low sodium chloride (NaCl) concentrations increase the sensitivity of the cAMP assay (Kasagi, Konishi, Iida *et al.,* 1982; Kasagi, Hidaka, Hatabu *et al.,* 1989; Kosugi, Mori and Imura, 1989) acting through a number of mechanisms as described in Chapter 5.

iv) Methods to determine growth

The difficulty in interpreting evidence for and against the existence and significance of TGIs lies in the variability of the methods used to detect them *in vitro* (Zakarija and McKenzie, 1990). The methods employed to study growth include [^{3}H]-thymidine ([^{3}H]-TDr) incorporation into DNA, mitotic arrest assays, direct cell counting, and cytochemical assays such as Feulgen densitometry and glucose-6-phosphate dehydrogenase. The variability and insensitivity and non-specificity of each method has been reported previously (Drexhage, Bottazzo, Doniach *et al.,* 1980). Major investigations into humoral factors causing growth in non-immunogenic forms of thyrotoxicosis are summarised in Table 1.05.

The use of [^{3}H]-TDr incorporation into DNA is commonly used as an indicator of cell growth. Unfortunately this technique is not specifically associated with cell growth alone and can reflect DNA repair without increase in cell number (Zakarija and McKenzie, 1990). Sources of error have been reviewed by Maurer (1981) and are summarised below:

The chemical and physical properties of [^{3}H]-TDr

- Impurities that bind to macromolecules within the cytoplasm may lead to false positives if methods such as autoradiography are used to detect [^{3}H]-TDr incorporation into DNA.
- Radiation from [^{3}H]-TDr can sometimes result in aberrations to the cell and even cell death without incorporation into DNA, leading to false negative results.

Factors influencing labelling of nuclear DNA

- The most commonly used [^{3}H]-TDr is in the form of [^{3}H]-methyl-5-TDr which can degrade over periods of days and transfer its methionine group to proteins other than DNA.
- Cells may synthesise DNA at a reduced rate if already in S-phase.

Thymidine incorporation into cells as a marker for cell division, therefore, results mainly in false positives. The method is useful as a screening test but should be used in conjunction with more

direct assessment of cell proliferation such as cell counting or cytochemical methods if a growth response is demonstrated.

Cytochemical assays such as the Feulgen reaction to identify cells in S-phase may be more sensitive. This assay was developed for use in guinea pig thyroid cells which reach a peak S-phase activity in 3 to 5 hours (Zakarija and McKenzie, 1990). However, most mammalian cells do not reach S-phase for 8 to12 hours and division may not be detected.

Wilders-Truschnig, Drexhage, Leb *et al.* (1990) demonstrated cell growth in FRTL-5 cell lines in response to purified IgG from human endemic goitre patients, using a mitotic arrest assay. A mitotic spindle poison was added to cells to accumulate metaphases. These cells were then stained with Giemsa and cells in metaphase were counted and expressed as an index of mitosis. This method is more time-consuming but possibly more specific for growth.

A combination of [^{3}H]-TDr incorporation and direct cell counting was used as confirmation of growth in FRTL-5 cells by Brown, Keating, Livingstone *et al.* (1992) following growth stimulation by purified IgG from the sera of hyperthyroid cats. Of all methods used, it would appear that the combination of cell counting with thymidine incorporation makes the determination of cell growth most accurate.

Table 1.05: Investigations of non-immunogenic thyrotoxicosis for TGIs

Investigators	Major Diseases Investigated	Cell System	Method of IgG Purification	Growth Assay	Effect
Drexhage, Botazzo, Doniach *et al.* (1980)	Graves' disease, TNG and others	Guinea-pig thyrocytes	Ammonium sulphate precipitation	Feulgen reaction for S-phase cells	Graves' disease positive TNG some positive
Chiovato, Hammond, Hanafusa *et al.* (1983)	Graves' disease and non-toxic nodular goitre	Intact rat thyroid follicles	Ammonium sulphate precipitation	^{3}H-thymidine	Graves' disease positive Non-toxic goitre some positive but less potent
Valente, Vitti, Rotella *et al.* (1983)	Graves' disease, TNG, subacute thyroiditis	FRTL-5	DEAE and ammonium sulphate precipitation	^{3}H-thymidine	Graves' disease positive, 2/7 nodular goitres positive, occasional normal control positive
McMullan and Smyth (1984)	Graves' disease and TNG	Guinea-pig thyrocytes	PEG and ammonium sulphate precipitation	NADPH assay	Graves' disease positive TNG some positive but less potent
Wiener and van der Gaag (1985)	TNG	Guinea-pig thyrocytes	Not specified	Feulgen reaction for S-phase cells	7/9 positive
van der Gaag, Drexhage, Wiersinga *et al.* (1985)	EG and TNG	Guinea-pig thyrocytes	Ammonium sulphate precipitation	Feulgen reaction for S-phase cells	43/62 TGI positive
Wilders-Truschnig, , Drexhage, Leb *et al.* (1990)	Endemic and sporadic goitre	FRTL-5	Staphlococcal Protein A	Mitotic arrest assay	Both endemic and sporadic positive
Brown, Keating, Livingstone *et al.* (1992)	Feline TNG	FRTL-5	DEAE	^{3}H-thymidine	13/21 cats positive for TGI
Vitti, Chiovato, Tonacchere *et al.* (1994)	Graves' disease and endemic goitre	FRTL-5	DEAE and ammonium sulphate precipitation	^{3}H-thymidine	Graves' disease positive EG negative

DEAE, diethylaminoethyl; EG, euthyroid goitre; TNG, toxic nodular goitre; PEG, polyethylene glycol;

c) ARGUMENTS AGAINST THE ROLE OF AUTO-ANTIBODIES IN NON-IMMUNOGENIC HYPERTHYROIDISM

Graves' disease is known to result from the presence of humoral factors which lead to the stimulation of growth and function of thyrocytes and is regarded as a classic example of an autoimmune disease. The existence of immunoglobulins which affect thyrocyte growth, but not function, in diseases other than Graves' disease has lead to much debate and will be summarised here. These conditions include endemic and sporadic goitre and nodular toxic and non-toxic goitre. The pathophysiology and pathogenesis of toxic nodular goitre in humans and cats is discussed in more detail in Chapter 1.04.

i) Human studies

The role of autoantibodies in non-immunogenic forms of thyrotoxicosis (such as Hashimoto's thyroiditis, toxic and non-toxic multinodular goitre) was investigated by Drexhage, Bottazzo, Doniach *et al.* (1980). They confirmed the existence of thyroid stimulating immunoglobulins *in vitro* in sera from human patients with Graves' disease and Hashimoto's thyroiditis which inhibited binding of radiolabelled TSH to its receptor. Later, using nucleic acid cytophotometry and [^{3}H]-TDr uptake to monitor growth, they failed to show any significant response to sera from 'non-autoimmune thyroid disease' (adenomas and toxic nodular goitre).

Using [^{3}H]-TDr uptake and intracellular cAMP accumulation, Valente, Vitti, Rotella *et al.* (1983) demonstrated that TGIs from Graves' disease and Hashimoto's thyroiditis stimulated growth, but IgG from Graves' disease in remission, nodular goitre, sub-acute thyroiditis or atrophic thyroiditis did not. They showed that three distinct Graves' disease groups existed: those with potent cAMP stimulation along with growth promoting activity, those with potent cAMP stimulation but low grade growth activation and those with potent growth promotion but low level cAMP production. They speculated that these immunoglobulins could act at different sites of the TSH receptor selecting for growth or cAMP production independently.

Peter, Gerber, Studer *et al.* (1986) reported that when nodules from human toxic nodular goitre patients were transplanted into nude mice, they continued to grow and remain functional. They

concluded that if humoral stimulants were involved, they were not necessary for the maintenance of the condition and a continuous potent humoral stimulation, as found in Graves' disease, was therefore not occurring in toxic nodular goitre. Once transformed, a lack of regulatory control or a positive stimulus must be exerted in these cells for them to continue to hypersecrete thyroid hormones.

Evidence for a lack of a regulatory element was suggested by Van Sande, Lamy, Lecocq *et al.* (1988), who reported the presence of a protein substrate (20KD) which was phosphorylated in the presence of TSH in tissue adjacent to nodular tissue but not in nodular tissue from autonomous thyroid nodules from human patients. They postulated that the absence of the 20KD protein could represent a lack of a negative controlling element and, hence the development of autonomy.

ii) Feline studies

Peter, Gerber, Studer *et al.* (1987), using their model from human studies, transplanted goitres from cats into nude mice and showed that they continued to grow and remain functional. Additionally, feline goitre cells behave differently in cell culture compared to normal feline thyroid tissue. Cells from toxic nodular goitres do not take up and organify ^{131}I in response to TSH, as occurs in normal thyroid tissue. This suggests an intrinsic alteration at the cellular level which leads to autonomy (Peter, Gerber, Studer *et al.,* 1991).

Neither Peterson, Livingstone and Brown (1987) or Kennedy, Thoday and Mooney (1989) found any evidence for the existence of TSIs in cats with hyperthyroidism using FRTL-5 cells and cAMP accumulation as a marker. Peterson, Livingstone and Brown (1987) measured intracellular cAMP as an indicator of second messenger stimulation in response to purified IgG from hyperthyroid cats. This group reported that no rise in cAMP occurred when comparing hyperthyroid cats to controls, concluding that TSIs were not involved in stimulating intracellular cAMP, and hence, the disease was not analogous to Graves' disease of humans. Using unpurified sera from hyperthyroid cats, Kennedy, Thoday and Mooney (1989) also found no increase in ^{131}I uptake when comparing hyperthyroid cat sera to sera from healthy feline

controls in FRTL-5 cells. By contrast, sera from six Graves' patients consistently increased iodine uptake.

d) ARGUMENTS FOR THE EXISTENCE AND SIGNIFICANCE OF THYROID GROWTH STIMULATING IMMUNOGLOBULINS IN NON-IMMUNOGENIC HYPERTHYROIDISM

ii) Human studies

Weiner and van der Gaag (1985) reported the existence of TGIs in a patient with Plummer's disease (localised thyroid autonomy) and suggested that autoimmune thyroid disease should be regarded as a spectrum where each patient was unique. This lead to speculation that TGIs exert a chronic but mild stimulation, perhaps priming the thyrotoxic event or selecting out populations of cells that have enhanced growth potential.

Similarly, van der Gaag, Drexhage, Wiersinga *et al.* (1985), using primary cultures of guinea-pig thyroid and purified IgG, found that TGIs in patients with euthyroid non-endemic goitre stimulated growth (measured by DNA cytophotometry). However, approximately 10 times more IgG was required to reach maximal responsiveness when compared to Graves' disease IgG.

ii) Feline studies

Brown, Keating, Livingstone *et al.* (1992) provided evidence that the serum of some hyperthyroid cats contained an immunoglobulin that binds to the TSH receptor and stimulated thyroid cell growth. This group purified IgG using the batch DEAE cellulose method. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) confirmed that the preparation was pure IgG. Using both [^{3}H]-TDr incorporation into DNA and direct cell counting, test sera, as a group, stimulated FRTL-5 cell growth by a factor of 15 over that from control animals.

Brown's group used two methods to demonstrate that this purified IgG was binding to the TSH receptor. Firstly, the IgG growth stimulating activity was inhibited completely by the addition of a

potent TSH receptor blocking antibody from a patient with Hashimoto's thyroiditis. Secondly, the purified immunoglobulin significantly inhibited binding of ^{125}I - bovine TSH to porcine thyroid membranes. No rise in intracellular cAMP was demonstrated, suggesting that another second messenger pathway, possibly the PIP cascade, may have been responsible for growth.

The majority of these studies in both humans and cats have tested the hypothesis that stimulatory immunoglobulins bind at or near the TSH receptor and activate it, causing thyroid cell growth and hyperfunction. Although this is true of the potent TSIs found in Graves' disease and Hashimoto's thyroiditis, the chronic low grade stimulation produced by immunoglobulins in other 'non-immunogenic' thyrotoxicoses may not be demonstrated by the relatively insensitive or inappropriate methods so far employed. Furthermore, it is likely that even if there is a role for TGIs in mitogenesis of cells in non-immunogenic goitrous disease, these are likely to be low grade and possibly intermittent or short lived, unlike the potent and chronic stimulation seen with Graves' disease immunoglobulins.

1.04 INTRODUCTION TO FELINE THYROTOXICOSIS

a) GENERAL INTRODUCTION

Feline hyperthyroidism is the most commonly diagnosed endocrine disease of cats (Thoday, 1988; Thoday and Mooney, 1992a) and in some areas appears to be the commonest disease of old cats *per se*. The clinical syndrome is well-characterised and documented in the veterinary literature. Despite cats being the only non-human species to develop spontaneous hyperthyroidism with any frequency, the close clinical and pathological resemblance to human toxic nodular goitre has been largely ignored and few investigations into possible causal triggers, or the pathogenesis of the disease, have been carried out.

After the two initial reports of the condition (Cotter, 1979; Peterson, Johnson and Andrews, 1979) the prevalence of feline hyperthyroidism increased rapidly, probably beyond what may be explained by improved awareness of, and diagnostic techniques for, the condition, prompting suggestions that this may truly be a new disease (Broussard, Peterson and Fox 1995). A retrospective survey of 500 cats from the Animal Medical Center, New York, from 1970 to 1984 found that less than 2 per cent of cats had gross evidence of thyroid enlargement at post-mortem examination. The present incidence at the same institution is approximately 3.3 per cent of cats at post-mortem examination (Broussard, Peterson and Fox 1995) but this increase is likely to be artificially low as most cats are now successfully treated.

The disease occurs exclusively in middle to old aged cats. The age at presentation varies from 6 years (Hoenig, Goldschmidt, Ferguson *et al.,* 1982; Peterson, Kintzer, Cavanagh *et al.,* 1983; Thoday and Mooney, 1992a) to 22 years (Theran and Holzworth, 1980; Theran,1981; Peterson, Kintzer, Cavanagh *et al.*, 1983), with a mean age of 12.8 years (Peterson, Kintzer, Cavanagh *et al.*, 1983) to 13 years (Thoday and Mooney, 1992a). There is no female sex predisposition (Peterson, Kintzer, Cavanagh *et al.*, 1983; Thoday and Mooney, 1992a) as there is with the three most common causes of hyperthyroidism (Graves' disease, toxic multinodular goitre and toxic solitary thyroid nodule) in humans (Toft, Campbell and Seth, 1981).

Only one epidemiological study has been published investigating risk factors that may be causal for feline toxic nodular goitre (Scarlett, Moise and Rayl, 1988). There was a significant association with the disease and exposure to flea sprays and powders, herbicides, consumption of canned food and an indoor lifestyle. Siamese cats had a ten-fold reduced incidence of the disease (Scarlett, Moise and Rayl, 1988). Feline immunodeficiency virus (FIV) was studied as a possible risk factor in a New Zealand study, although no correlation between a positive FIV status and development of hyperthyroidism was found (Jones, Hodge and Davies, 1995).

b) CAUSAL PATHOLOGY

Two main forms of thyrotoxicosis occur in humans, with Graves' disease accounting for approximately 60 per cent and nodular and multinodular goitre 30 per cent. The remaining 10 per cent result from viral thyroiditis, Hashimoto's thyroiditis and other, rarer forms. Despite some debate over classification of the histological changes in the thyroid of affected cats, 98 to 99 per cent are reported as "thyroid adenomas (adenomatous hyperplasia)" (Peterson and Becker, 1983). These cases thus closely resemble clinically and pathologically toxic nodular or multinodular goitre of humans, where adenomatous functioning nodules hypersecrete thyroid hormones independently of TSH production (Studer and Gerber, 1991). Thyroid adenocarcinomas do not result in thyrotoxicosis in humans (Braverman and Utiger, 1991). In contrast to this, although an uncommon disease, feline thyroid adenocarcinoma causes 1 to 2 per cent of cases of feline hyperthyroidism (Peterson, Kintzer, Cavanagh *et al.*, 1983). Graves' disease has not been reported in cats although comparable histological changes have been described (Peter, Gerber, Studer *et al.*, 1986).

c) THYROID AUTONOMY AND THE PATHOGENESIS OF HUMAN TOXIC NODULAR GOITRE

Given that TSH positively controls the function, expression of differentiation, and growth (in some species) of thyrocytes, it is not difficult to imagine that somatic mutations of the TSHR or its coupled G protein activating subunit ($G_{s\alpha}$) would result in development of a monoclonal group of thyrocytes or "toxic adenoma" (Vassart, Desarnaud, Duprez *et al.*, 1995).

Seventeen such "gain-of-function" somatic mutations have been described in adenomas from human toxic nodular goitre (Ledent, Parma, Dumont *et al.*, 1994; Parma, Van Sande, Swillens *et al.,* 1995; Van Sande, Parma, Tonacchera *et al.*, 1995; Tonacchera, Van Sande, Cetani *et al.*, 1996; Tonacchera, Van Sande, Parma *et al.*, 1996; Duprez, Hermans, Van Sande *et al.*, 1997; Holzapfel, Fuhrer, Wonerow *et al.*, 1997; Parma, Duprez, Van Sande *et al.,* 1997; Uyttersport, Allgeier, Baptist *et al.,* 1997) 13 of which occur within codons 480 to 640 of the TSHR. Activating mutations of the TSHR constitute the major cause of adenomas, accounting for about 80% of cases (Parma, Van Sande, Swillens *et al,* 1995). The variability in location and frequency of these mutations is dependent on scientific methods and the population studied (Tonacchera, Cetani, Van Sande *et al.*, 1996). The most important sites for functional mutations appear to be in the first and second extracellular loops, the third intracellular loop, and the third, sixth and seventh transmembrane segments (Parma, Van Sande, Swillens *et al.*, 1995). A particular mutation of alanine 293 confers a constitutively active receptor. This demonstrates that the wild type TSHR is kept in a constrained configuration with a low cAMP turnover (Dremier, Delange, Vassart *et al.,* 1996). Another hot spot for mutation appears to be Phe 631 (Paschke, Van Sande, Parma *et al.*, 1996). When transfected into COS-7 cells, these TSHR mutations lead to an increase in TSH-independent cAMP production over wild type (Duprez, Parma, Van Sande *et al,* 1994; Paschke, Tonacchera, Van Sande *et al.*, 1994; Paschke, Van Sande, Parma *et al.*, 1996). The mutation thereby increases not the maximal, but the basal, activity of the receptor (Dremier, Delange, Vassart *et al.,* 1996). This is compatible with the observation that both *in vivo* and *in vitro*, thyroid hyperfunctioning adenomas can be stimulated (to varying degrees) by exogenous TSH and that *in vitro,* the ratio of maximally enhanced to basal cAMP is lowered (Dremier, Delange, Vassart *et al.,* 1996). The mutations do not usually (Van Sande, Parma, Tonacchera *et al.*, 1995) but may also activate the PIP pathway (Parma, Van Sande, Swillens *et al.*, 1995; Ledent, Denef, Cottecchia *et al.*, 1997). Mutations in the extracellular loops seem active in this latter respect. The activation of the receptor is highly individual. Some TSHR mutations appear maximally activated, whilst others respond similarly to stimulation by TSH compared to that of the wild type cell.

Mutations of the TSHR and its associated G-protein lead to autonomy from TSH. Other mechanisms that lead to autonomy, and hence increased function or growth of the thyrocyte, may result from any factor which leads to increased accumulation of cAMP within the cell. This

may be caused by increased production or decreased breakdown of cAMP. Autonomy may also result from non-genetic mechanisms and still be hereditary. This mechanism is known as epigenicity (Dremier, Delange, Vassart *et al.*, 1996). This occurs when cells secreting autocrine or paracrine factors lead to increased cAMP accumulation by themselves or neighbouring cells. As thyrocytes also have a minimal constitutive activity unlike other endocrine cells, an increase in thyroid mass may also lead to autonomy.

Autonomy leads to hyperfunction only if the volume of adenoma is large enough, and/or the iodine concentration of the diet is adequate or excessive. A second consequence of autonomy is decreased sensitivity to the inhibitory effects of iodide. Indeed, administration of iodide to patients with hyperfunctioning adenoma may sometimes lead to hyperthyroidism (Cooper, 1996).

d) PATHOGENESIS OF FELINE THYROTOXICOSIS

Peter, Gerber, Studer *et al.* (1987) demonstrated that feline nodular goitres are truly autonomous when transplanted into nude mice. For this reason, it would appear that a humoral signal is not important for the continuation of the disease. Thyroid growth stimulating immunoglobulins could, however, initiate the disease, perhaps by selecting out faster growing or more autonomous thyrocytes, which eventually reach 'critical mass' when thyroid hormones are produced in excess of the animal's requirements and thyrotoxicosis then ensues. As in the human literature, the presence and significance of TGIs has been debated. This has been previously discussed in Chapter 1.03.

Somatic TSHR mutational analysis in the pathogenesis of feline toxic nodular goitre is described in this thesis in Chapter 6 and has been published separately (Pearce, Foster, Imrie *et al.*, 1997).

e) PATHOPHYSIOLOGICAL EFFECTS OF THYROTOXICOSIS IN THE DOMESTIC CAT

Clinical signs mimic those of human thyrotoxicosis. Most cats exhibit moderate to severe weight loss despite polyphagia, tachycardia, irritability and restlessness, intermittent vomiting and diarrhoea, and polydipsia and polyuria (Thoday and Mooney, 1992a). In 88 (Hoenig,

Goldschmidt, Ferguson *et al.*, 1982) to 98 per cent of cases (Peterson, Kintzer, Cavanagh *et al.*, 1983; Thoday and Mooney, 1992a), palpable goitre is present. Weight loss, which may be profound, is caused by a number of pathophysiological mechanisms. There is an intermittent decreased food intake due to vomiting, rapid gastrointestinal motility leads to malabsorption and metabolic rate increases as a direct effect of thyrotoxicosis. Behavioural changes such as irritability, restlessness and aggression are mainly due to increased sympathetic drive. Polyuria and polydipsia may result from a number of causes including increased metabolic and glomerular filtration rates (reviewed by Thoday and Mooney 1992a).

As in human thyrotoxicosis, the effects of thyroid hormone excess are expressed in numerous body systems. Cardiac disease is common and many cats present with tachycardia (a heart rate of 240 beats per minute or greater), gallop rhythms, arrhythmias and murmurs. Reversible hypertrophic cardiomyopathy associated with thyrotoxicosis is a common feature. Cats may present in congestive heart failure (Thoday and Mooney, 1992a). These effects are thought to result from a variety of mechanisms including interactions between thyroid hormones and the sympathetic nervous system, direct effects of thyroid hormones on the heart, and a cardiac response to increased tissue demand for oxygen (reviewed by Thoday and Mooney, 1992a).

Common gastrointestinal tract manifestations include polyphagia with intermittent short periods of anorexia (Peterson, 1984). Vomiting, diarrhoea, and increased frequency of defaecation (Peterson, 1982) are seen frequently. Thyroid hormones are thought to have a direct emetic action on the chemoreceptor trigger zone (Rosenthal, Jones and Lewis, 1976) but other contributory factors may include rapid overeating (Peterson, Kintzer, Cavanagh *et al.*, 1983) and increased intestinal motility (Papasouliotis, Muir, Gruffydd-Jones *et al.*, 1993). This may be a possible mechanism for diarrhoea and increased frequency of defaecation as well as contributing to the weight loss seen with this disease.

As the effects of thyrotoxicosis are expressed in many body systems, biochemical changes occur commonly and include elevations in serum alkaline phosphatase (SAP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea and creatinine. ALT is considered liver specific in cats and the serum increase in hyperthyroidism reflects a mild hepatopathy due to increased metabolic rate and hepatic lipidosis in these animals. An increase

in total SAP may reflect elevations in a number of serum iso-enzymes although the bone and liver iso-enzymes predominate in humans (Thoday and Mooney, 1992a).

f) DIAGNOSIS OF FELINE THYROTOXICOSIS

In most cats, the definitive diagnosis of feline hyperthyroidism rests on the detection of elevated serum concentrations of circulating total T4 (TT4). Serum total triiodothyronine (TT3) concentrations are variably elevated. In addition, the T3 suppression test or thyrotropin releasing hormone (TRH) stimulation test are sometimes used to confirm cases with reference range or slightly elevated serum TT4 concentrations. TSH assays are not currently available for the cat.

g) TREATMENT OF FELINE THYROTOXICOSIS

Hyperthyroidism requires treatment to prevent severe weight loss and cardiac disease which may result in fatal cardiac failure. There are currently three therapeutic options for cats with thyrotoxicosis: long term use of an antithyroid drug, surgical thyroidectomy and radioactive iodine (^{131}I) treatment. Management with radioactive iodine is very effective but its use is restricted to specialised licensed centres only four of which are, at present, available in the U.K. Long-term medical management often results in inadequate control, frequently associated with poor owner compliance (Mooney, Thoday and Doxey, 1992). The treatment of choice for most veterinary practitioners, therefore, is surgical thyroidectomy.

As hyperthyroid cats are poor anaesthetic risks, the induction of euthyroidism by prior short-term medical therapy dramatically reduces peri-surgical mortality (Peterson, Kintzer, Cavanagh *et al.*, 1983; Birchard Peterson and Jacobson, 1984; Peterson and Turrel, 1986). The drugs of choice are carbimazole (CBZ)(NeoMercazole, Roche) available in Europe and methimazole (Tapazole, Eli Lilly) available in the U.S.A. In both humans (Jansson, Dahlberg and Lindstrom, 1983) and cats (Peterson and Aucoin, 1993), CBZ is converted to methimazole, by which it exerts its effects *in vivo*. Methimazole is subsequently concentrated within the thyroid gland and inhibits thyroid peroxidase-catalysed reactions, subsequently blocking iodide oxidation, thyroglobulin iodination and coupling of iodinated residues (Trepanier, 1990). Propylthiouracil (PTU), a pyrimidine from the thioureylene family, is no longer used in cats due to the high incidence of haematological

complications (Peterson, Hurvitz, Leib *et al.*, 1984). These drugs do not result in ablation of thyroid tissue and do not interfere with uptake of iodine by the thyroid gland or the release of preformed hormones (Kintzer, 1994). For this reason, the hyperthyroid state returns within 24 to 72 hours of drug withdrawal. These drugs are also known to have immunomodulating effects in humans reducing anti-thyroid antibody titres. As there is no conclusive evidence that there is an immune component to thyrotoxicosis in cats, the relevance (if any) of immunomodulating effects by this class of drugs in this species is unknown.

Mild clinical adverse reactions (anorexia, vomiting and lethargy) requiring drug withdrawal occur with CBZ and methimazole. However, CBZ appears to be slightly better tolerated with a withdrawal requirement of approximately 8 per cent of treated cases (Mooney, Thoday and Doxey, 1992) compared with approximately 15 per cent of cases treated with methimazole (Peterson, Kintzer and Hurvitz, 1988). Mild and transient leucopenia and lymphocytosis, not requiring drug withdrawal, have been reported in 5 per cent of cats treated with CBZ (Mooney, Thoday and Doxey, 1992). Additional, potentially life-threatening effects such as thrombocytopenia and agranulocytosis, have been reported in up to 3 per cent of cats treated with methimazole (Peterson, Kintzer and Hurvitz, 1988) but, not to date, in cats treated with CBZ. Other reported adverse effects of methimazole include mild haematological abnormalities (eosinophilia, lymphocytosis, and slight leucopenia), usually occurring within the first 2 months of therapy, and the development of positive antinuclear antibodies (ANA) in 16.4 per cent and 21.8 per cent of treated cats respectively. Positive antinuclear antibodies was not associated with the development of immune-mediated disease.

Other drugs that have been used to treat feline hyperthyroidism include stable iodine (^{127}I), beta-adrenoreceptor blocking agents, either alone, together or in combination with the thioglyoxalines (reviewed by Thoday and Mooney, 1992b; Mooney and Thoday, 1999) and calcium ipodate (Murray and Peterson, 1997).

Stable iodine was the earliest agent used to effectively treat human thyrotoxicosis. Large doses dramatically decrease thyroid hormone synthesis (the Wolff-Chaikoff effect) by reducing peroxidase-catalysed organification of iodide (Wolff and Chaikoff, 1948). Thyroid hormone release is also decreased as a result of inhibition of thyroglobulin endocytosis. Unfortunately, the

failure of thyroid function of most patients to normalise (Emerson, Anderson, Howard *et al.*, 1975) and the rapid escape from its inhibitory control (Cooper, 1991) means that iodine cannot be used as sole therapy for long-term management of thyrotoxicosis. In humans, stable iodine may also reduce the vascularity and friability of the thyroid gland allowing more easy manipulation at the time of surgery in patients previously treated with thioureylenes (Chang, Wheeler, Woodcock *et al* ., 1987) or with beta-adrenoreceptor blocking agents (Feek, Sawers, Irvine *et al.*, 1980) but not when used alone (Coyle and Mitchell, 1982). It has been previously recommended for this purpose prior to surgical thyroidectomy in cats (Thoday and Mooney, 1992b).

Pharmaceutical preparations of both potassium iodide and iodine are currently unavailable in the U.K. Potassium iodate (85mg tablets yielding 50mg free iodine; Cambridge Self-Care Diagnostics) is now used as the source of stable iodine in human medicine and has the advantage of a longer shelf-life.

It is currently believed that an excess of thyroid hormones causes an increase in the number of beta-adrenoceptors or an amplification of the adrenergic signal at the cell membrane level (Trepanier, 1990) and that many of the signs of hyperthyroidism are due to subsequently enhanced catecholamine activity with increased sympathetic drive. Thus, the beta-adrenoreceptor blocking drugs very effectively ameliorate many of these signs and some studies (Feely and Peden, 1984; Lennquist, Jorsto, Anderberg *et al.*, 1985; Alderberth, Stenstrom and Hasselgren, 1987) have reported them to be as effective as the thioureylenes in the pre-surgical management of human patients awaiting thyroidectomy. However, the negative nitrogen balance, increased cardiac output and elevated rate of oxygen consumption of thyrotoxicosis are seldom normalised by beta-blockade (O'Malley, Abbott, Barnett *et al.*, 1982) and patients remain hyperthyroid.

Propranolol, the beta-adrenoreceptor blocking drug most frequently used in hyperthyroid cats, has been employed as the sole agent for presurgical stabilisation (Carlson, 1986). It is a non-selective β_1- and β_2-adrenoreceptor blocking agent with no direct effects on the thyroid gland. However, like some other beta-adrenoreceptor blockers, propranolol also decreases peripheral monodeiodination of T4 to T3 (Feely and Peden, 1984). Nevertheless, serum TT3 concentrations

fall by a maximum of 30 per cent, serum TT4 concentrations are usually unaffected and euthyroidism is not restored (Lotti, Delitala and Devilla, 1977; Wiersinga and Touber, 1977).

Feek, Sawers, Irvine *et al.* (1980) showed that a combination of propranolol and potassium iodide (as a source of stable iodine) induced clinical and biochemical euthyroidism in all 10, human Graves' disease patients prior to thyroidectomy. In successfully treated patients, both the serum TT3 and TT4 concentrations fell to their reference ranges within means of 5 and 8 days respectively. There was also a significant fall before surgery, and a transient rise after surgery, of rT3. A secondary rise or 'escape' of thyroid hormones was seen in only two (20 per cent) of patients. The authors suggested that there may be a previously unrecognised synergism between the drugs and that the combination may be the optimal preoperative preparation for patients with Graves' disease.

It is clear, therefore, that an alternative method of preparation for surgery is required for a relatively small percentage, but, because of the high incidence of the disease, a very significant number, of hyperthyroid cats. In the study described in Chapter 8, two groups were chosen to examine, initially, the independent action of both propranolol and potassium iodate and subsequently their combined effect.

1.05 SELENIUM AND SELENOENZYMES

a) SELENIUM

Selenium was discovered 180 years ago because of its toxicological properties in livestock (Foster and Sumar, 1997). Most forms of selenium are metabolised to selenite or are further reduced to selenides (Foster and Sumar, 1997). Animals receive selenium in food as selenoamino acids (selenomethionine and selenocysteine) and as methylated/nonmethylated selenium. Selenomethionine and selenocysteine are absorbed across the gut using the amino acid transport proteins. Organic forms of selenium (e.g. selenomethionine and selenocysteine) are more readily retained by tissues (Foster and Sumar, 1997), and increase plasma selenium more quickly and reaches higher concentrations than inorganic forms (selenite and selenate) (Neve, 1998). In contrast to this, platelet glutathione peroxidase (GPX) activity is increased more rapidly by inorganic than organic forms of selenium (Neve, 1998). Cellular uptake of selenium occurs by a number of mechanisms which vary depending on the form of selenium ingested. Absorption of selenium from the digestive tract is not a limiting factor in bioavailability (Daniels, 1996). It appears that selenium is absorbed better with a high protein diet (Daniels, 1996) but for unknown reasons, the availability of selenium from absorption in fish, especially tuna, is lower than other foods (Daniels, 1996).

Selenium is contained in most bodily tissues at an average concentration of 0.2µg/g associated with tissue proteins either loosely bound or as selenium analogues of sulphur and amino acids which form selenoproteins (Foster and Sumar, 1997). The first selenoprotein to be identified was cytoplasmic GPX (cGPX). It functions (with vitamins A, C and E) as a cellular protector against oxidative damage by catalysing the breakdown of H_2O_2 and fatty acyl lipid peroxides in the presence of reduced glutathione.

Regions of the world with low soil selenium include Denmark, eastern Finland, areas of northeastern and south central China and parts of the United Kingdom (Aberdeenshire). Less severe deficiencies occur in areas of Canada, western Australia, New Zealand and the U.S.A. (Foster and Sumar, 1997).

b) ASSESSMENT OF SELENIUM STATUS

Measurement of selenium concentration in whole blood or its fractions, and measurement of its functional action [such as GPX activity in erythrocytes (RBCs)] are the most commonly used measures of selenium status. Selenium supplementation results in increases in activity of RBC GPX activity up to a plateau that serves as a measure of the optimal daily requirement (Neve, 1998). Because of this, RBC GPX is of no value in monitoring high intakes of selenium. Only whole blood or plasma selenium concentrations are reliable for this. The discovery of other forms of GPX (plasma GPX [p-GPX], phospholipid hydroperoxidase [phGPX] and gastrointestinal) and other selenoenzymes ID and thioredoxin reductase (TR) may serve as new areas of investigation regarding the re-assessment of the amounts of selenium required for optimal daily intake (Neve, 1998). Nail and hair selenium status may also be a reasonable indicator of long-term selenium supplementation. Because of the relatively long half-life of RBCs, plasma selenium concentration measurements are also far more sensitive than the RBC selenium concentration to acute changes in selenium status (Neve, 1998). RBC GPX is therefore used to indicate medium-term selenium status and plasma GPX, short-term selenium status.

Intakes of selenium for humans range from 20 to 30 μg/day in low selenium areas (in Keshan intake is less than 11μg/day), to in excess of 1500μg/day in high selenium areas. The recommended daily allowance for selenium intake in humans (55μg/day and 70μg/day for females and males respectively) may reflect an underestimation of selenium requirement considering that some functional characteristics of selenium status have not been reached at these levels of intake (Neve, 1998). At intakes of 800μg/day, discolouration of the nails occurs (Neve, 1998) and therefore 400μg/day is considered the maximal safe dietary intake.

c) SELENIUM DEFICIENCY

Selenium deficiency has been linked to a number of disease states in humans such as cancer, muscular dystrophy, malaria, cardiovascular disease (Keshan disease), alopecia areata, and pregnancy hypertension syndrome (Foster and Sumar, 1997; Ximin, Zhongxi, Wenkang *et al.,* 1998) as well as increasing the virulence of viruses (Beck, Shi, Morris *et al.,* 1995). Keshan

disease is a well-characterised disease of selenium deficiency and is associated with the development of chronic cardiac insufficiency, cardiomegaly, gallop rhythm and arrythmias. Increased risk of atherosclerosis has also been associated with selenium deficiency. This has been postulated to occur through the poor expression of free radical scavenging selenoproteins which detoxify oxidised lipids (such as low density lipoproteins) and lipid peroxides (Foster and Sumar, 1997). In animals, selenium deficiency has been associated with white muscle disease in pigs, and other livestock (Oldfield, 1987). Other diseases reported include pancreatic atrophy in chickens, and abnormalities of spermatozoa and cataract formation in rats (Oldfield, 1987).

d) SELENIUM TOXICITY

The dietary concentrations required to cause selenium toxicity in animals vary due to the type of selenium ingested, type of diet and species (Foster and Sumar, 1997). Selenosis in animals can result in disease of the integument (hoof rot and alopecia). In humans, toxicity has been reported to induce hair and fingernail loss and peripheral neuropathies (Foster and Sumar, 1997).

e) SELENOENZYMES REGULATING THYROCYTE GROWTH AND FUNCTION

There is considerable evidence in a number of species to suggest that selenium status may modify thyroid function and peripheral thyroid hormone metabolism (Delange, 1996; Beech, Walker, Beckett *et al.*, 1995; Howie, Walker, Akesson *et al.,* 1995). Conversion of T4 to the more metabolically active hormone T3 is catalysed by the selenoenzyme IDI. Synthesis of T4 may also be regulated through modified expression of the selenoenzyme E-GPX (Howie, Walker, Akesson *et al.*, 1995). There is evidence to suggest that expression of certain selenoproteins also appears to correlate with thyroid cell growth both *in vivo* and *in vitro* and protect from oxidative damage (Howie, Arthur, Nicol *et al.,* 1998).

When there is adequate iodine supply, the rate limiting step in thyroid hormone synthesis is the amount of H_2O_2 present at the apical membrane of the thyrocyte. Degradation of H_2O_2 in the follicular lumen is regulated by E-GPX, and intracellular GPX detoxifies H_2O_2 and thus protects the thyrocyte from its harmful oxidant effects. It has been suggested that E-GPX is an important

regulator of thyroid hormone synthesis in the human thyrocyte (Howie, Walker, Akesson *et al.*, 1995). In humans, stimulation of the PIP pathway increases H_2O_2 synthesis and decreases secretion of E-GPX into the follicular lumen thus leading to increased H_2O_2 concentrations in this space. E-GPX is a glycoprotein which comprises four identical subunits each having a molecular mass of 24 000 and is functionally, immunologically and structurally different from cGPX. In rats and humans the major source of E-GPX in plasma is the renal proximal tubules but other tissues have been identified as excretors such as the placenta and bronchoepithelial tissues (tissues under high oxidative stress) (Sunde, 1994).

i) Selenium and iodine status reflect thyroidal pathology - endemic goitre

In areas of the world where goitre is described as 'endemic' (greater than 10 per cent of children are affected), two forms of endemic cretinism (myxoedematous and neurological) exist. In these areas, the incidence of the disease may be as high as 15 per cent of the population (Delange and Ermans, 1996). Endemic cretinism is by far the most serious complication of iodine deficiency (Delange, 1996). Affected individuals have subnormal intellect and physical development. Myxoedematous cretinism is used to describe severe hypothyroidism and stunted growth with a low incidence of goitre. Plasma T4 and T3 concentrations are low with an elevated plasma TSH concentration (Delange, 1996). Neurological cretinism is more common and describes mental deficiency which may be accompanied by neurological problems including hearing and speech deficits.

The pathogenesis of cretinism is only partially understood but involves iodine deficiency (Delange, 1996). Combined selenium and iodine deficiency appears to favour the development of myxoedematous cretinism. The proposed mechanism for this is that iodine deficiency results in stimulation of the thyroid by TSH which results in increased production of H_2O_2. In addition, there is a large increase in TSH secretion in the neonatal period which further enhances the production of H_2O_2. Selenium deficiency results in loss of cGPX expression and thus a loss of protection from the accumulation of H_2O_2. The end result is thyroid necrosis and fibrosis (Sunde, 1994; Contempre', Dumont, Denef *et al.*, 1995; Delange, 1996). There is still some debate about selenium- and iodine-deficiency being the only factors involved in the pathogenesis of myxoedematous cretinism as it is not the most predominant form of cretinism in some areas with

combined deficiency (Moreno, Suetens, Mathieu *et al.*, 1998). During iodine deficiency with adequate selenium intake, cGPX expression is induced in preference to phGPX. cGPX is much more efficient in metabolising H_2O_2 than other forms of glutathione peroxidase (Mitchel, Nicol, Beckett *et al.*, 1996).

ii) Thioredoxin reductase

Thioredoxin reductase (TR) is a dimeric selenoprotein comprising subunits of Mr 55 to 60 KDa, each having a selenocysteine residue near the carboxyl terminus (Holmgren, 1985; Beckett, Howie, Nicol *et al.*, 1998). Thioredoxin reductase reduces oxidised thioredoxin (a small multifunction and ubiquitous protein) (TRx-S_2) to reduced thioredoxin [TRx-$(SH)_2$] using an NADPH-dependent reaction. Both TR and thioredoxin have been shown to have growth regulating effects on cells (Berggren, Gallegos, Gasdaska *et al.*, 1996; Gallegos, Gasdaska, Taylor *et al.*, 1996; Gasdaska, Berggren, Berry *et al.*, 1999). Reduced thioredoxin is a powerful protein disulphide reductase [e.g. Trx-S_2 + NADPH + $H^+ \leftrightarrow$ Trx$(SH)_2$ + NADP ; Trx$(SH)_2$ + protein-$S_2 \leftrightarrow$ Trx-S_2 + protein$(SH)_2$]. TR can also detoxify lipid hydroperoxides and hydrogen peroxide (Beckett, Howie, Nicol *et al.*, 1998).

TR appears to have a greater capacity to detoxify H_2O_2 and lipid peroxides than GPX and is induced by the same second messenger pathways that induce H_2O_2 production. This may suggest that one important function of TR (at least in thyrocytes) is to protect the thyrocyte from oxidative damage from H_2O_2. The leucocyte, which produces H_2O_2, does not express TR but has a short life span, presumably as a result of oxidative damage.

Labelling experiments of human thyrocytes with ^{75}Se-selenite have demonstrated that at least eight major selenoprotein bands are expressed. Most have a Mr of between 14 and 31 KDa. The expression of human thyroidal TR has been shown to be increased by PMA and the calcium ionophore A23187 (Beckett, Howie, Nicol *et* al., 1998; Howie, Arthur, Nicol *et al.,* 1998). However, in FRTL-5 cells, PMA has little effect on TR expression, but the addition of PMA and A23187 produces a marked increase in TR expression. Addition of 8-bromo-cAMP and TSH have no effect on TR expression in human thyrocytes, suggesting that the calcium-PIP but not

the cAMP pathway are involved in the regulation of TR expression in humans (Howie, Arthur, Nicol *et al.,* 1998).

TR also appears to have an important role in cell proliferation. TR has been shown to act as an intracellular growth factor for both normal and tumour cells in culture (Berggren, Gallegos, Gasdaska *et al.*, 1996; Gallegos, Gasdaska, Taylor *et al.*, 1996) by activating transcription factors that lead to cell division (Powis, Gasdaska, Gasdaska *et al.,* 1997). To date, no TR binding sites have been found on the surface of cancer cells. TR also acts in an autocrine manner by enhancing the cellular response to other growth factors (Berggren, Gallegos, Gasdaska *et al.*, 1996; Gasdaska, Kirkpatrick, Monfort *et al.,* 1996; Powis, Gasdaska, Gasdaska *et al.,* 1997).

Selenium status can also modify TR expression/activity. TR activity is inhibited by high concentrations of selenite (Bjornstedt, Kumar and Holmgren, 1995) and, *in vivo* ,TR activity in rat liver, lung and kidney undergoes transitory increased expression when these animals are fed a high selenium diet (Berggren, Mangin, Gasdaska *et al.*, 1999). Changes in selenium status may, therefore, result in altered expression of this enzyme resulting in altered growth characteristics or changes in detoxifying capacity which may leave them exposed to oxidative damage. This may be particularly important in the thyrocyte where protection from harmful H_2O_2 is particularly important.

A number of TR enzymes have been sequenced from human placenta (Gasdaska, Gasdaska, Cochran *et al.*, 1995), Jurkat T cells (Gladyshev, Jeang and Stadtman, 1996) and lung adenocarcinoma cells (Tamura and Stadtman, 1996), rat neuroblastoma (Zhong, Arner, Ljung *et al.*, 1998) and bovine liver neuroblastoma (Zhong, Arner, Ljung *et al.*, 1998). Recently, it has been suggested that multiple forms of TR exist. A novel isoform of TR (named TR-β) has been cloned and shown to have 54 per cent identity to previously cloned human TR (Gasdaska, Berggren, Berry *et al.*, 1999). Similarly, two isoforms of TR from rat liver have also been cloned and sequenced (Lee, Kim, Kwon *et al.*, 1999).

A number of human primary cancers such as lung, colon, cervical and liver tumours have been shown to over-express thioredoxin. Transfection with reduced thioredoxin can increase tumour

growth and inhibit apoptosis (Gasdaska, Berggren, Berry *et al.*, 1999). The thioredoxin reductase system thus plays an important role in cell growth and death (Gasdaska, Berggren, Berry *et al.*, 1999).

f) HIERARCHY OF SELENIUM SUPPLY

In rats and humans, the thyroid contains more selenium (per gram weight) than any other tissue suggesting an important role for the trace element in this organ. There is an important hierarchy of selenium supply to tissues such that in selenium deficiency, the thyroid, brain and skin retain the trace element, whilst it is rapidly lost from the liver, kidneys and muscle (Arthur, Nicol and Beckett, 1993; Beckett, Beech, Nicol *et al.,* 1993; Calomme, Vanderpas, Francois *et al.*, 1995; Larsen and Berry, 1995). Thyroidal IDI activity is maintained in times of selenium deficiency in the rat, whilst hepatic IDI activity falls (Beech, Walker, Beckett *et al.,* 1995). In addition, there is also an important hierarchy of selenium supply to different selenoenzymes within tissues. Thyroidal cGPX expression diminishes in times of selenium deficiency whereas thyroidal IDI expression increases (Howie, Arthur, Nicol *et al.,* 1998). This suggests that thyroidal IDI-I has an important role in the generation of T3 in certain species and this has to be retained in selenium deficiency.

g) SELENIUM STATUS IN CATS

Concentrations of selenium in some commercial cat foods is recommended at 0.01 to 0.04 mg/400KCal (personal communication 1999, Waltham). The selenium status of domestic cats has not been previously studied. It is, therefore, difficult to determine if selenium status or selenoenzymes play a role in feline hyperthyroidism and whether selenium status may be manipulated in order to prevent this important disease. As high selenium intake can regulate apoptosis and selenoenzymes such as TR can modify growth of cells, it is possible that the selenium status of cats may be an important factor in the development of feline thyrotoxicosis. Because of the marked geographical distribution of hyperthyroidism in cats, it is possible that an environmental toxin or lack of an environmental or dietary protector (such as selenium), may result in effects within the feline thyrocyte that lead to autonomy of the thyroid gland and the disease which we describe as feline toxic nodular goitre. Additionally, selenium has been shown

to promote apoptosis and it is this mechanism which is thought to be involved in the cancer-protective effects of this trace element (McCarthy, 1998).

h) IODINE STATUS IN CATS

Whilst daily iodine requirements of cats have been published, they are based purely on speculation as no specific studies have been conducted. The iodine content of cat food has been shown to be very variable. Mumma, Rashid, Shane *et al.* (1986) showed that it could contain up to 10 times the recommended daily intake, possibly due to the inclusion of thyroid glands from slaughtered animals. In another study, Johnson, Ford, Tartellin *et al.* (1992) reported that two brands of commercial cat food contained excess amounts of iodine whilst nine contained less than the recommended intake. They speculated that a wide variation in iodine intake may be responsible for thyroid dysfunction in the cat. One major pet food company regulates iodine content in cat foods to provide adult cats with approximately 0.1 to 4.5mg of iodine per cat per day (personal communication 1999, Waltham).

In a related study, Tartellin, Johnson, Cooke *et al.* (1992) observed that an increase in dietary iodine resulted in increased urinary excretion of iodine and a reciprocal drop in serum FT4 concentration. This study confirmed that the Wolff-Chaikoff effect occurs in cats. The experimental period was too short to draw any conclusions on the long term effects of iodine over-supplementation.

The only short to medium term published study investigating chronic dietary iodine deficiency or excess in cats did not show any significant differences in serum FT4 concentrations over a 5 month period (Kyle, Tartellin, Cooke *et al.*, 1994). Unfortunately, this group did not measure any other indicators of thyroid status (e.g. T3, rT3, or TSH concentrations) so it is impossible to speculate as to the significance of their findings regarding other aspects of thyroid function. Additionally, the study was probably not long enough to make any conclusions regarding iodine deficiency as it would take longer than 5 months to deplete a cat of iodine.

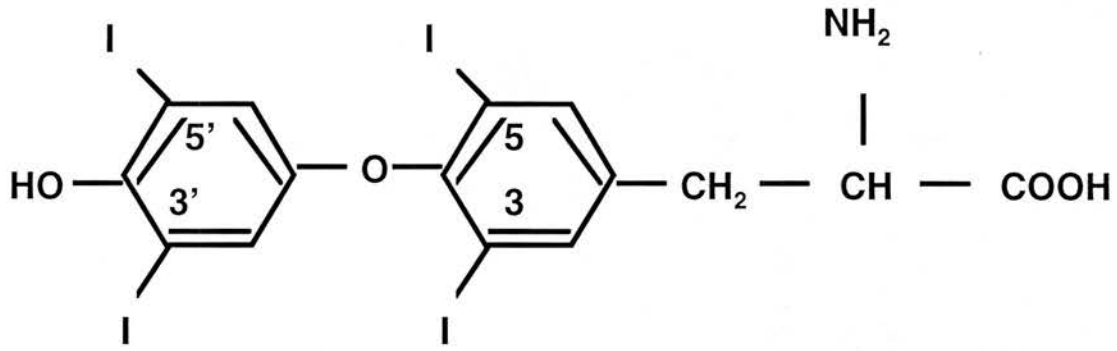
In addition to iodine causing suppression of thyroid hormone synthesis (Wolff-Chaikoff effect), iodine has effects on thyrocyte growth and immunity. Iodine deficiency leads to increased

thyrocyte growth *in vitro* (endemic goitre). In humans with Hashimoto's thyroiditis, the administration of excess dietary iodide may result in hypothyroidism (due to permanent Wolff-Chaikoff inhibition) in greater than 60 per cent of patients (Roti and Vagenakis, 1996). When patients from iodine deficient areas are given iodine supplementation an increase in hyperthyroidism occurs. This is not noted in nonendemic euthyroid goitre areas. Most of these patients were considered to have pre-existing, clinically silent, multinodular goitre and it appears that the administration of iodine unmasked thyroid autonomy (Roti and Vagenakis, 1996). Iodine intake also regulates the pathological type of thyroid disease that occurs in humans. In iodine deficient areas, the percentage of cases of autonomously functioning thyroid nodules is increased compared to that of iodine replete areas (Hay and Morris, 1996). Patients with Graves' disease are easier to control in times of iodine deficiency (Roti and Vagenakis, 1996). High iodine intake is also known to modify the action of growth factors such as transforming growth factor-β (Roti and Vagenakis, 1996).

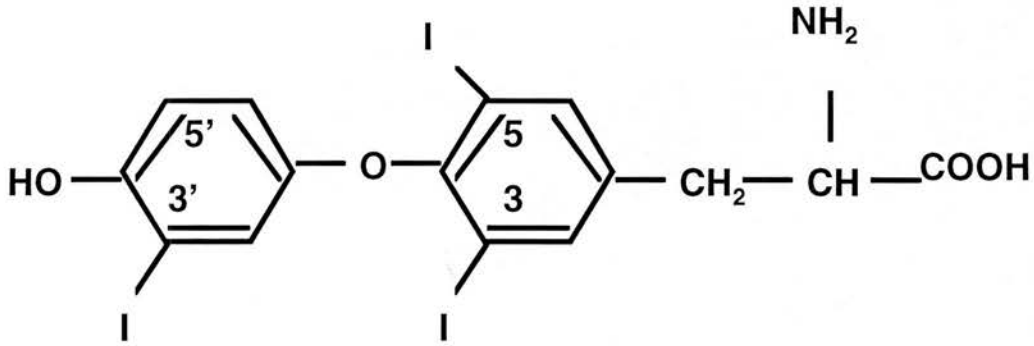
Studies in animals suggest that iodine has an important role in the development of autoimmune thyroid disease. Iodine administration has been shown to induce lymphocytic thyroiditis in hamsters, beagles, mice, certain strains of rats and chickens (Roti and Vagenakis, 1996). The pathogenesis of this autoimmune thyroiditis may include changes in the immunogenicity of iodine-rich thyroglobulin, cellular damage from free radicals, direct cytotoxic effects of iodine or changes in regulation of the MHC class I (Roti and Vagenakis, 1996). In contrast to this, Wistar rats made iodine deficient have been shown to develop autoimmune thyroiditis (Roti and Vagenakis, 1996). Controversy occurs in the relationship of iodine intake and the development of Hashimoto's thyroiditis, some studies have reported an increase in incidence with increased iodine intake, other studies demonstrate no correlation (Roti and Vagenakis, 1996).

In vitro, FRTL-5 cells show inhibited growth with the addition of pharmacological concentrations of iodine (Roti and Vagenakis, 1996), whereas feline thyrocytes isolated from cats with multinodular goitre do not show any change in growth characteristics (Aeschimann, Gerber, Von Grunigen *et al.*, 1988).

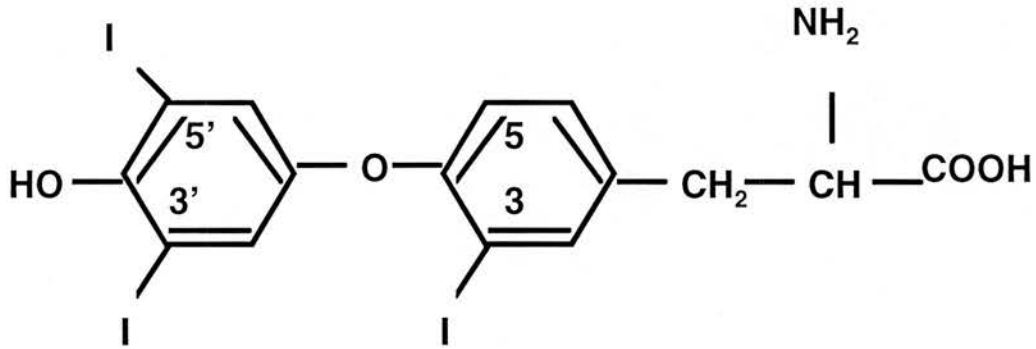
There is, therefore, a strong possibility that dietary factors such as selenium and iodine may play a role in the development of feline toxic nodular goitre.



3,5,3',5'-tetraiodo-L-thyroxine (thyroxine, T4)



3,5,3'-triiodo-L-thyronine (T3)



3,3',5'-triiodo-L-thyronine (reverse T3)

Figure 1.01: Chemical structure of the three iodothyronines: 3,5,3',5'-tetraiodo-L-thyroxine (thyroxine, T4), 3,5,3'-triiodo-L-thyronine (T3), 3,3',5'-triiodo-L-thyronine (reverse T3).

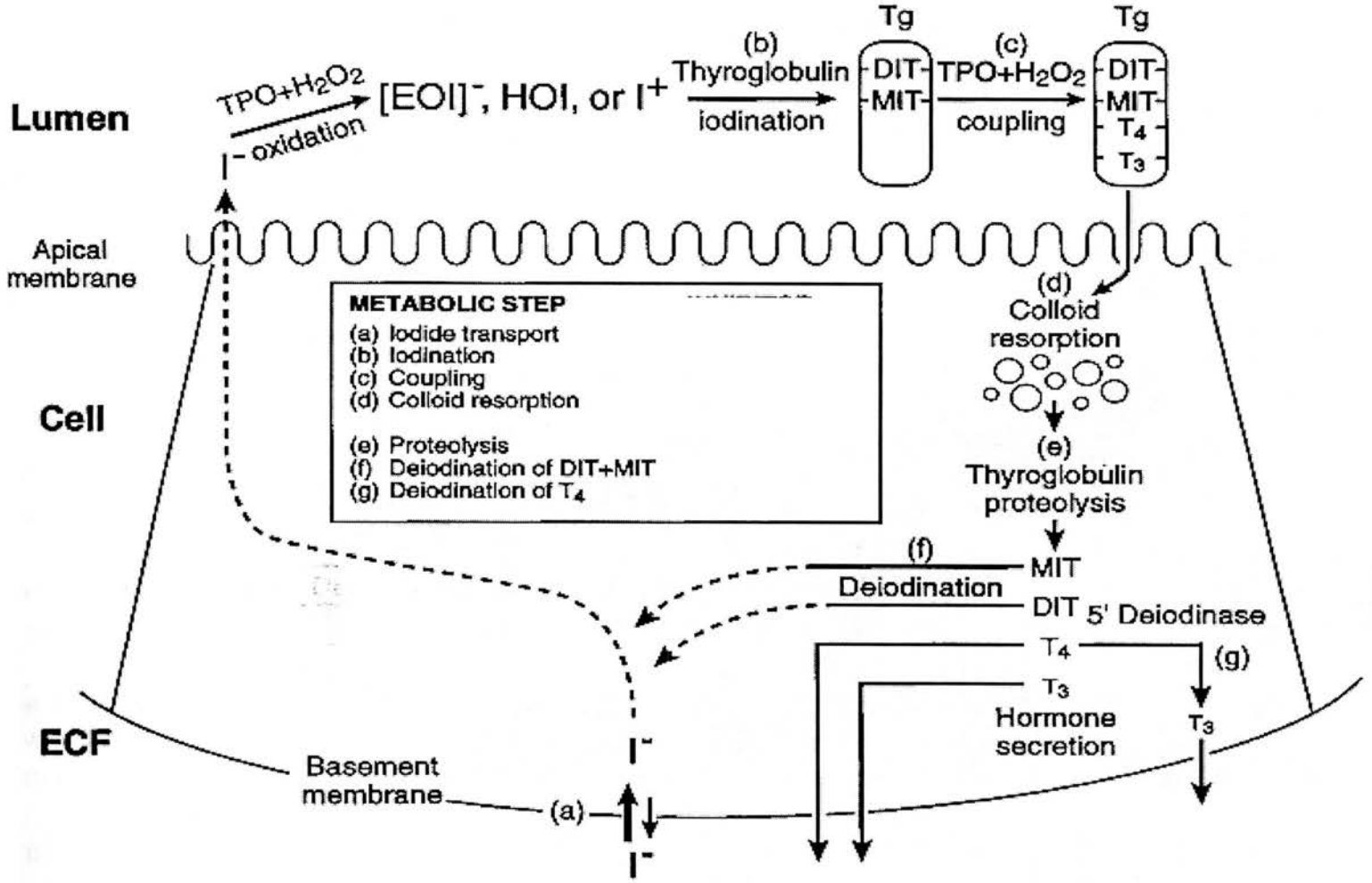


Figure 1.02: Thyroid hormone synthesis. DIT, diiodotyrosine; ECF, extracellular fluid; MIT, monoiodotyrosine; TPO, thyroperoxidase (Taurog, 1996)

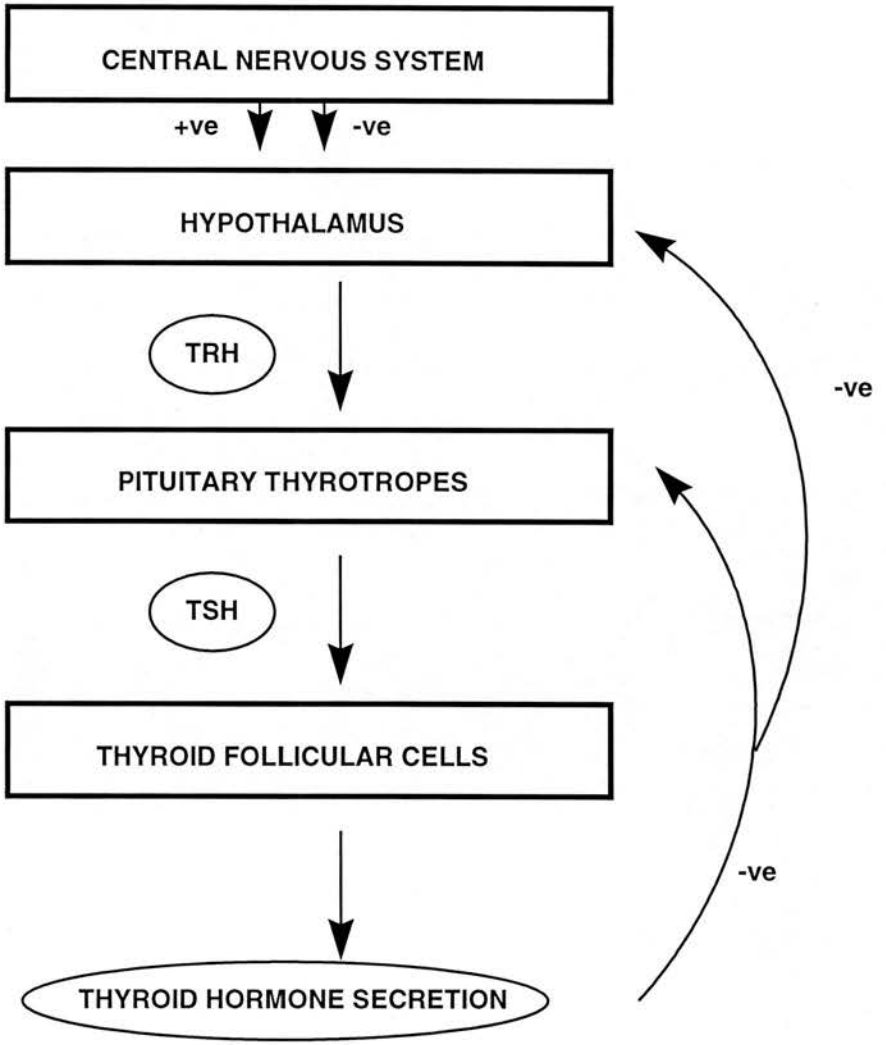


Figure 1.03: The hypothalamic-pituitary-thyroid axis. TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone

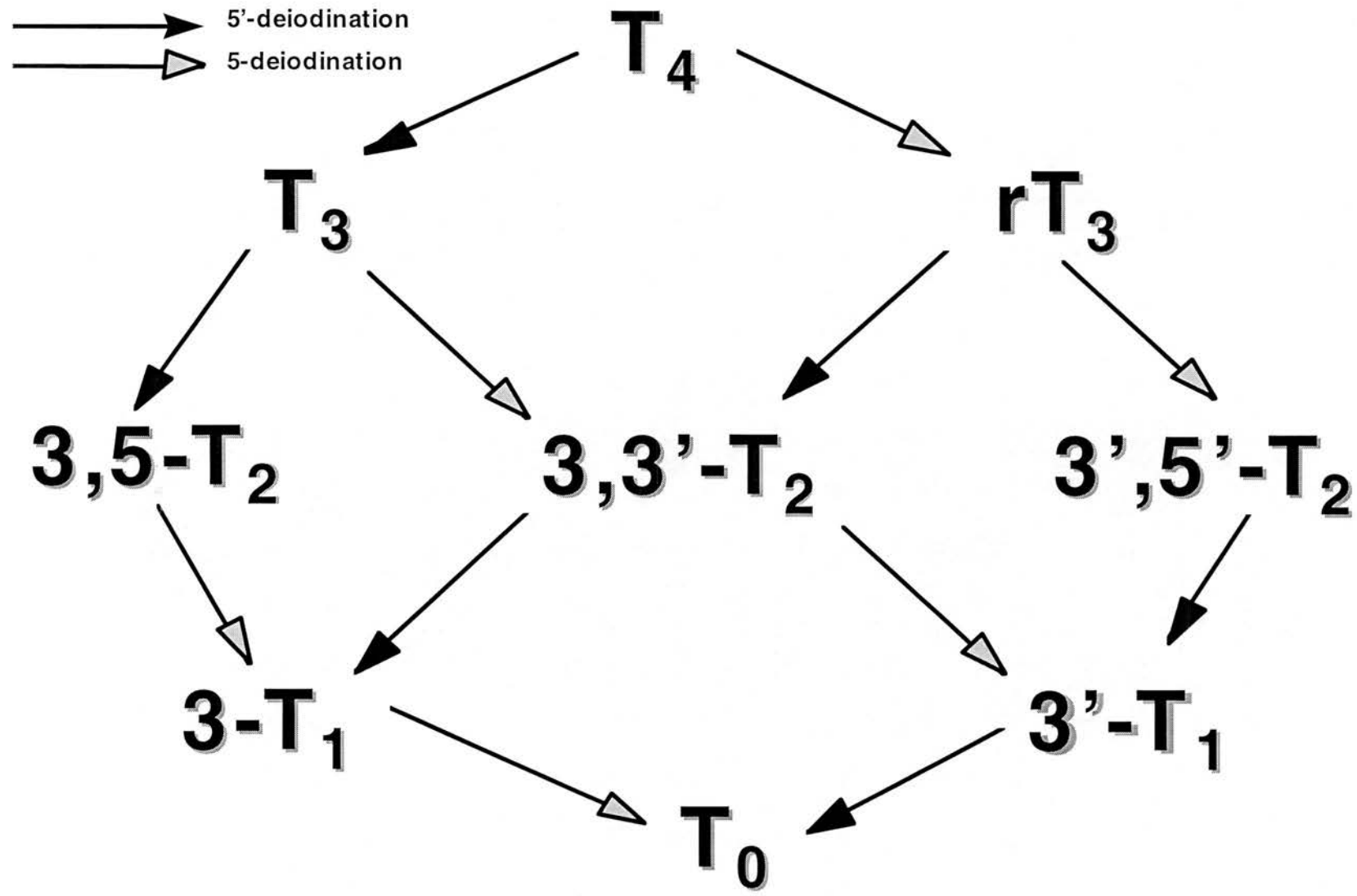


Figure 1.04: Pathways of thyroid hormone deiodination

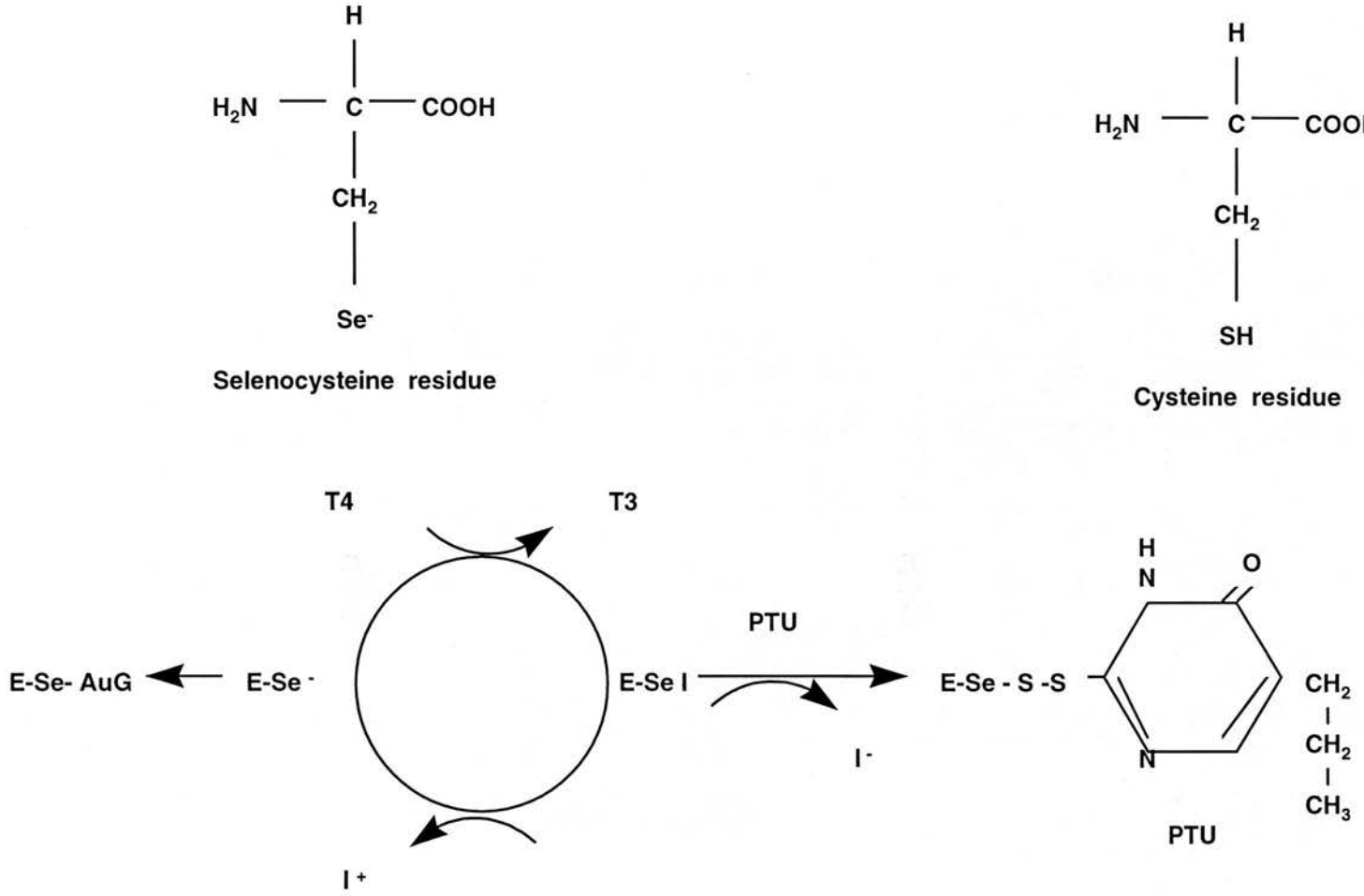


Figure 1.05: Selenocysteine residue of type I iodothyronine deiodinase (IDI) and proposed mechanism of inhibition by propylthiouracil (PTU) and gold (AuG). E, type I iodothyronine deiodinase (IDI); Se, selenocysteine residue; I, iodine

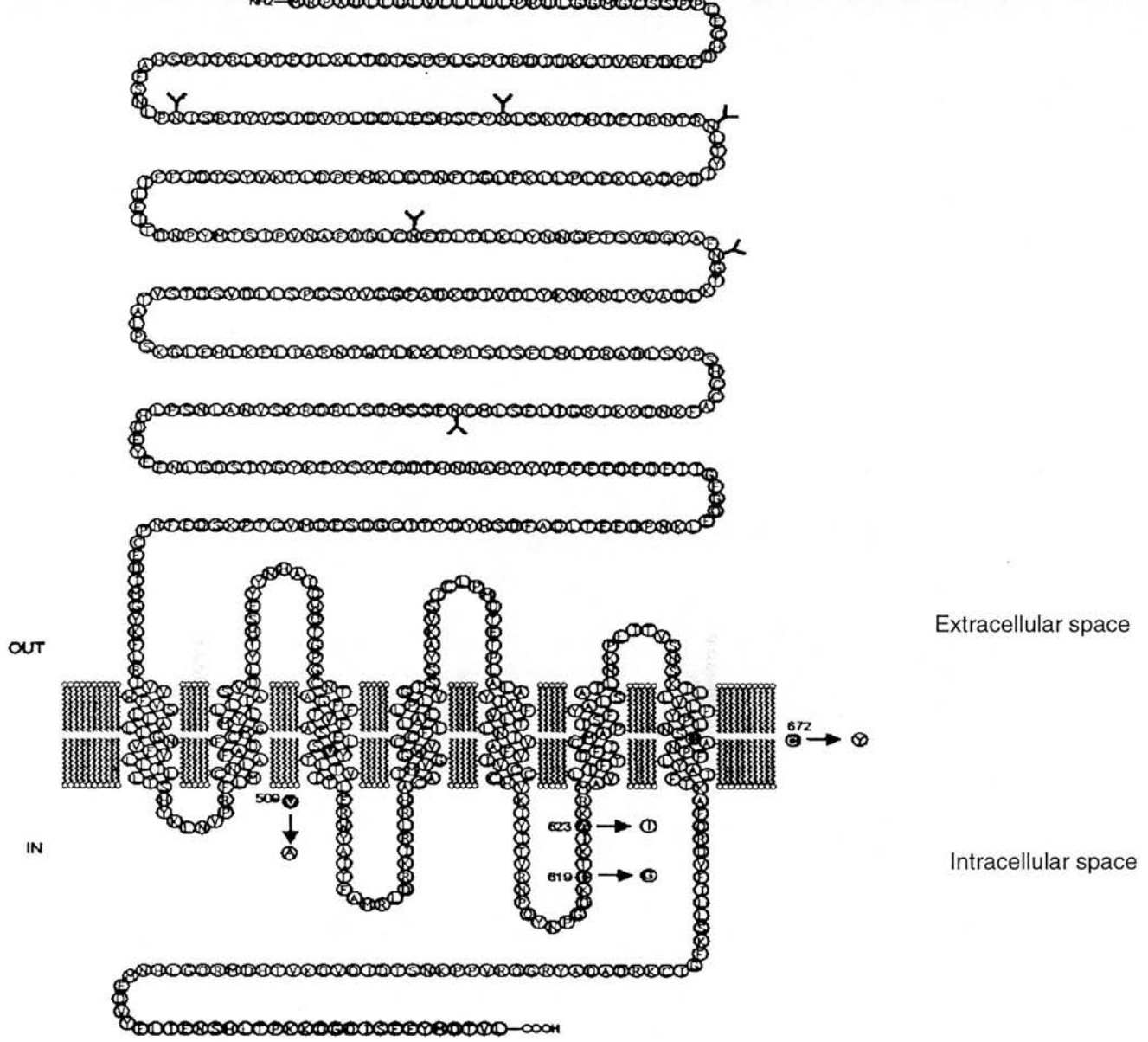


Figure 1.06: Molecular structure of the human thyrotropin hormone (TSH) receptor illustrating extracellular and intracellular domains, and transmembrane segments (From Ludgate and Vassart, 1995).

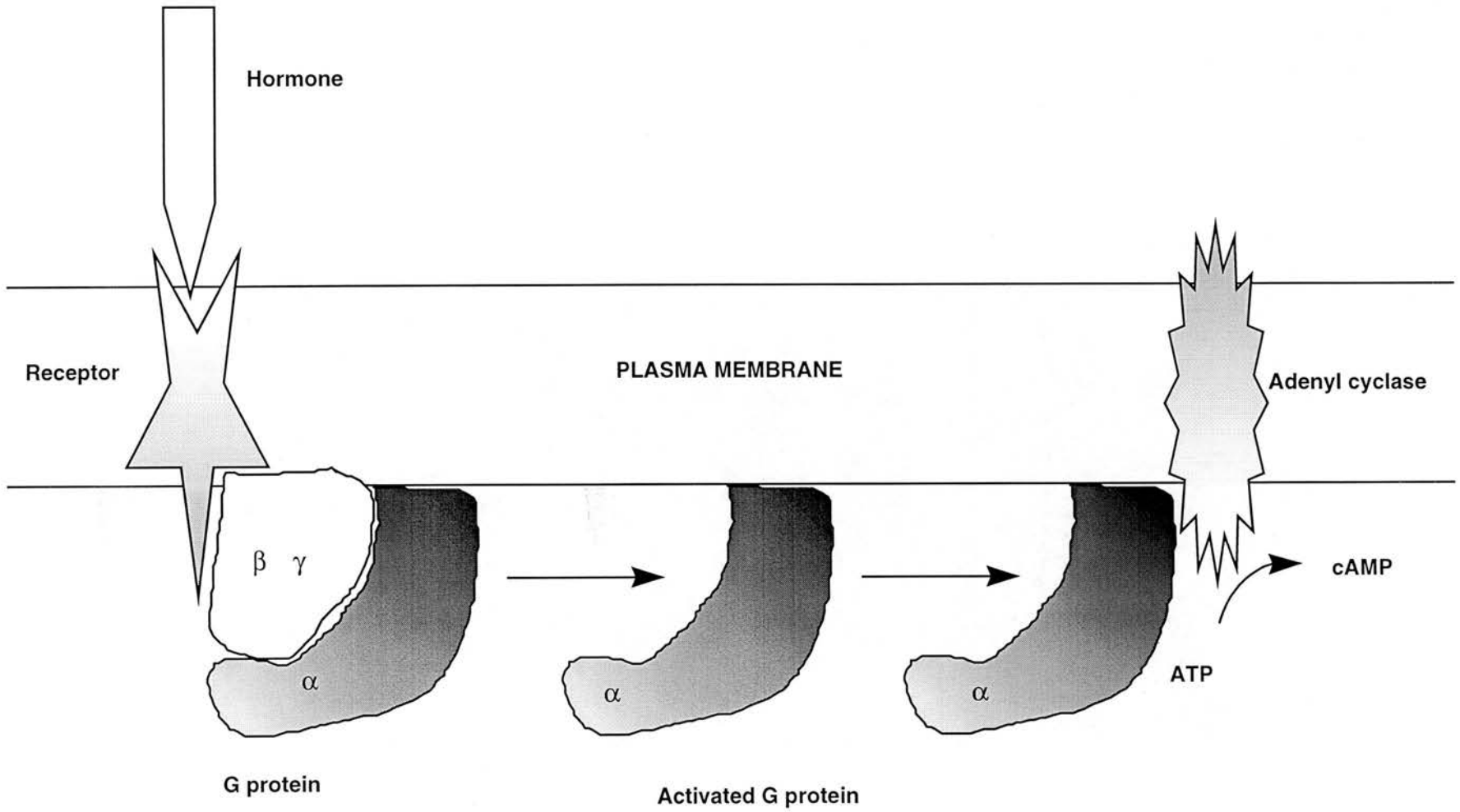


Figure 1.07: Hormonal activation of adenyl cyclase through G protein dissociation. Binding of hormone to receptor promotes dissociation of the alpha subunit to stimulate adenyl cyclase which catalyses the conversion of ATP to cAMP. ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate.

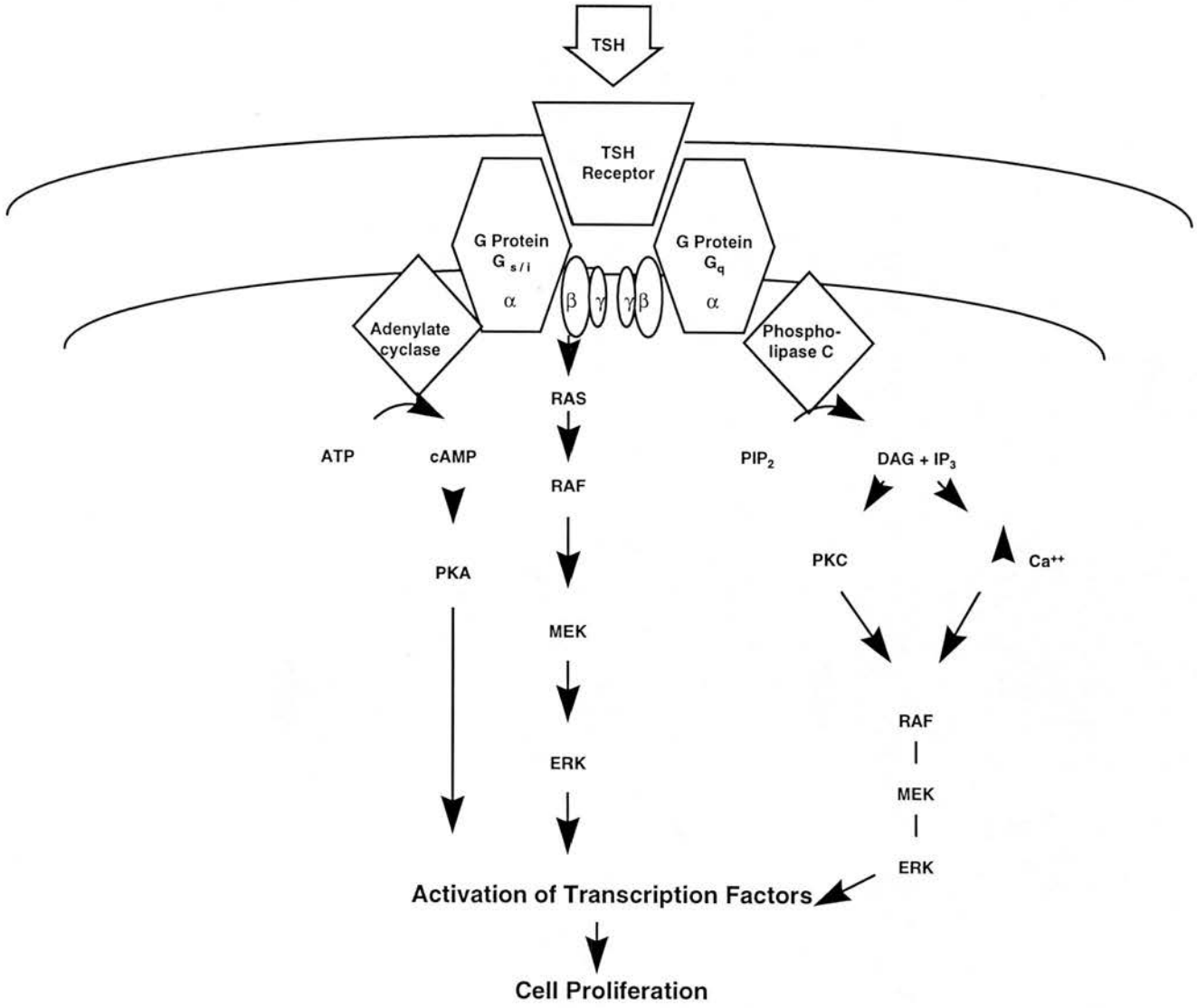


Figure 1.08 : Schematic model for illustration of second messenger systems in the thyrocyte and the regulation of cell proliferation. G protein activation generates both alpha-specific and beta/gamma-specific signals. ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5 triphosphate; MEK, mitogen-activated extracellular-signal regulated kinase; PKA, cAMP dependent protein kinase. (Adapted from Clark *et al.,* 1989; Dhanasekaran*et al.,* 1998).

2.00 MATERIALS AND GENERAL METHODS

2.01 CHEMICAL SUPPLIERS

The following products were purchased from the suppliers listed below.

Amersham International plc, Buckinghamshire, UK.

myo- [^{3}H]-Inositol 1.0mCi/ml (81.0Ci/mmol; 435mCi/mg); [*Methyl* - ^{3}H]-Thymidine 1mCi/ml (25Ci/mmol; 102mCi/mg).

Bio-Rad Laboratories, Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, U.K.

Glass plates, low range molecular weight markers; N,N,N,'N'-tetramethyl ethylenediamine (TEMED).

Bio-stat diagnostics, Cheshire, UK.

Reverse -T3 radioimmunoassay kit (125 tubes).

Boehringer Manheim, Lewes, East Sussex, UK.

Trypsin, Dispase (type II).

Calbiochem Novabiochem, Beeston, Nottingham, UK.

Insulin, sodium, bovine; Somatostatin -14.

DPC, Caernarvon, UK.

Total T4 Radioimmunoassay kit 100 tubes.

Gibco, Life Technologies, Paisley, U.K.

Earle's balanced salt solution (EBSS); Hank's balanced salt solution (HBSS).

Lockertex, Loker Wire Weavers Ltd, P.O Box 161, Church Street, Warrington, Cheshire, U.K.

Nylon gauze (100 and 30 μm).

MERCK, Leicester, U.K.

Acetic acid, dimethylformamide; ethanol, ethylenediaminetetraacetic acid (EDTA); glass fibre filters 25mm; hydrochloric acid (HCl); microcrystalline cellulose; polyethylene glycol (PEG); potassium phosphates (KPO_4, KH_2PO_4); sodium hydroxide (NaOH); trichloroacetic acid (TCA).

Millipore, Watford, U.K.

Centricon 10 centrifuge filters.

National Institute for Biological Standards and Control (NIBSC), Hertfordshire, U.K.

Bovine pituitary thyrotropin stimulating hormone (bTSH); insulin-like growth factor 1 (IGF-1).

NEN Life Sciences Products, Boston, USA

[^{125}I-rT_3], L-3,3',5'- triiodothyronine (reverse T3) specific activity > 28Mbq/µg.

Pharmacia Biotech, St Albans, U.K.

HiTrap protein G columns, 1 and 5ml; methylene bis-acrylamide.

Sigma Aldrich Co Ltd, Poole, Dorset, UK.

Acetic anhydride, ammonium persulphate; ampicillin sodium; apo-transferrin (human); aurothioglucose; bovine serum albumin powder (BSA); Brij; bromoacetic acid N-hydroxysuccinamide ester; bromophenol blue; Coon's F-12 modification powdered media; D-glucose; dialysis tubing cellulose membrane; DL-dithiothreitol; epidermal growth factor (EGF) (mouse); radiographic film Kodak X-OMAT; glycerol; gly-his-lys acetate; glycine hydrochloride; haemoglobin standard (18g/dl); Ham's F-12 Nutrient mixture; hydrocortisone; Igepal CA-630; 3-isobutyl-1-methylxanthine (IBMX); 2-mercaptoethanol; micrcrystalline cellulose; newborn calf serum heat inactivated; phorbol 12-myristate 13-acetate (PMA); propylthiouracil (6-n-propyl-2-thiouracil, PTU); RPMI-1640 Medium 10x; scintillant (Ultima gold); Sephadex (G25, LH20); sodium bicarbonate solution (7.5%); sodium dodecyl sulphate (lauryl sulphate, SDS); sucrose; L-thyroxine (T4); 3,3',5'-triiodo-L-thyronine free acid (rT3); triethylamine; Trypsin-EDTA solution 1x; Trizma (2-amino-2-hydroxymethyl propane-1,3-diol) hydrochloride.

2.02 MATERIALS OBTAINED FROM NON-COMMERCIAL SOURCES

i) Human serum and feline tissues

Euthyroid human serum (determined by a reference FT4 and FT3 and negative TRABs titre, used as negative controls and to stop reaction in the deiodinase assay was kindly supplied by Mr A. Jordon (Scottish Blood Transfusion Service, Royal Infirmary of Edinburgh). Feline tissues, thyroid glands obtained from hyperthyroid cats following thyroidectomy for the treatment of the disease and euthyroid cats euthanased for reasons other than thyrotoxicosis, and organs for IDI assay following euthanasia were obtained from colleagues at The University of Edinburgh's Small Animal Clinic and from the staff of veterinary clinics in Edinburgh and the rest of the United Kingdom as listed in the acknowledgements.

ii) Cell lines

FRTL-5 cells were purchased from the European Collection of Cell Cultures, Porton Down, Salisbury, UK. They were also kindly donated by Dr Steve Bidey of The University of Sheffield. Chinese hamster ovary cells (JPO9 and JPO2) were the kind donation of Professor Tony Weetman of the Rowett Research Institute, Aberdeen, UK.

2.03 GENERAL METHODS

2.031 Assay of IDI Activity In Tissue Homogenates and Microsomal Fractions By Release Of ^{125}I from ^{125}I-rT3.

i) Preparation of tissue homogenates and microsomal fractions

Tissues were homogenised in 100mMKH_2PO_4/1mMEDTA buffer pH 7.4, at a concentration of 20% w/v and subsequently centrifuged at 1500g for 10 minutes at 5°C. The supernatant was removed and 2ml sored at -80°C until assay was performed on this fraction. The rest of the supernatant was centrifuged at 12 000g for 20 minutes. The resulting supernatant was then centrifuged at 105 000g for 60 minutes to isolate the microsomal fraction. The microsomal pellet was resuspended in assay buffer and recentrifuged at 105 000g to wash the microsomes and resuspended in assay buffer to a concentration of 1µg/µl

ii) Assay procedure for tissue homogenates

For assay using crude tissue homogenates, 200µl of sample was incubated with 1000µl of purified tracer at an activity of 0.5µCi/ml in the presence of 5mmol/l dithiothreitol (DTT). Sequential experiments in which the reaction was stopped after 15minutes, 30 minutes, 60minutes, 5hours, and 24hours by the addition of 0.5ml of human sera (which binds rT3 and T2), were carried out and the protein was precipitated with 40% TCA. The sample was then centrifuged at 1500g for 10minutes and 0.8ml of supernatant removed. The total counts in 0.8ml of the the supernatant, remaining supernatant and pellet were determined and the percentage of ^{125}I released from ^{125}I-rT3 was calculated as follows :

[s ÷ (s+p)] x 100% corrected for blank. (Where p = pellet; s = supernatant)

1% conversion was considered to represent 4.6 fmols of deiodinated rT3

2.032 Assay of IDI Activity In Tissue Homogentates Using T4 as Substrate

50mg of T4 was diluted in 10ml absolute ethanol and a working solution of T4 made by diluting 1ml of stock T4 in 100ml of 0.25M sucrose/0.05M Tris/1mM EDTA buffer pH 7.4 with 3% BSA. Tissue homogenates were prepared as discussed previously in the aforementioned buffer with

20mM DTT added. Homogenates were spun at 1500g for 10 minutes to remove cell debris. 2ml of the resulting supernatant was then preincubated for 10 minutes to reach 37°C. 200µl of T4 was added to start the reaction. At each designated time point, 200µl of sample was removed in duplicate and placed into tubes containing 400µl of absolute ethanol and centrifuged at 1500g for 5 minutes. T3 in the supernatant was then assayed by chemiluminescence after dilution of the sample 1:10 with buffer without DTT. Controls were buffer with no T4, homogenates with no T4, and homogenates with T4 at time zero.

2.033 Affinity Labelling of IDI-1 in Microsomal Liver Fraction With N-bromoacetyl [^{125}I]-rT3

200µl of ^{125}I-rT3 was evaporated to dryness under a stream of dry nitrogen and 20µl of 1.5mg/ml bromoacetic acid n-hydroxy-succinamide ester in dimethylformamide was added to the dried tracer. To this, 5µl of 10% (v/v) triethylamine in dimethylformamide was subsequently added and the mixture left at room temperature for 50 minutes. The iodinated affinity label was then purified by passing it through a LH20 Sephadex column (2ml) equilibrated with 0.1mol/L HCl. The label was eluted with 95% ethanol and stored at -20°C until used (within 7 days).

Microsomes from tissues were isolated as above (2.031) and diluted to 1mg/ml protein in 50mM TrisHCl, 3mM EDTA, 3mM DTT. A 50µl aliquot (50µg protein) of this fraction was added to 0.6µCi of N-bromoacetyl - [^{125}I]rT_3 affinity label (stored in ethanol), which had been evaporated to dryness under a steam of dry nitrogen and reacted for 15 minutes.

Samples were then diluted 1:3 with 'boiling mix' consisting of SDS (35mmol/L), glycerol (1.4mmol/L), 2-mercaptoethanol (0.3mmol/L) and bromophenol blue (15mmol/L) and heated for 10 minutes at 90°C.

To study the inhibition of BrAc[^{125}I]rT_3 labelling of IDI, 0.1mM propylthiouracil (PTU), or 12mM aurothioglucose (AuG) was added to each test. Samples were then loaded onto a sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (SDS-PAGE) gel and the proteins separated and visualised by autoradiography as described subsequently.

2.034 Sodium dodecyl/sulphate polyacrylamide-gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out at room temperature in gels consisting of a stacking gel (32 ml H_2O; 7.2 ml acrylamide (30% acrylamide, 0.8% bis-acrylamide); 20 ml 0.375M Tris/HCl pH6.8; 0.6 ml 10% SDS; 0.2 ml TEMED [N,N,N',N'-tetramethylethylenediamine]; 0.2 ml 10% ammonium persulphate) and a 14 cm long 12% resolving gel (17 ml H_2O; 32 ml acrylamide; 30 1M tris/HCl pH 8.85; 0.8 ml 10% SDS; 0.15 ml TEMED; 0.15 ml 10% ammonium persulphate).

Protein samples were prepared for electrophoresis by heating at 90°C for 10 minutes in "boiling mix" at a final protein concentration of 1mg/ml. Electrophoresis was performed using a Protean II electrophoresis system (Bio-Rad Laboratories Ltd, Watford, Herts, U.K) with a 0.3% Tris/1.44% Glycine 0.1% SDS electrode buffer through the stacking gel at 200V, 35mA 50 watts and 300V, 50mA, 50 watts through the resolving gel. The gel was stained in 0.2% (w/v) Coomassie Brilliant Blue R in a water/methanol/acetic acid (50:50:7, by volume) solution for 30 minutes and destained in two changes of a water/methanol/acetic acid (88:5:7 by volume) solution overnight.

The stained gel was then sandwiched between two sheets of distilled water pre-soaked drying film and dried under vacuum using a programmable gel drier. The molecular weights of the standard proteins were plotted against the distance travelled, and a standard curve drawn, which was used to determine the molecular weights of unknown proteins.

2.035 Autoradiography of SDS/PAGE gels

Autoradiography was performed on gels which had been used to run samples labelled with ^{125}I or ^{75}Se using Kodak X-OMAT XAR-5 x-ray film and consisted of placing the dried gels in close contact with the film in an exposure cassette. Gels were laid down at -70°C for 15 hours to 4 days to produce a sharp image. Films were kindly processed by the staff of the Department of Radiology, the Royal Infirmary of Edinburgh.

2.036 Protein Determination

All protein determinations were carried out using the methods descibed by Bradford (1976), adapted for automated use on a Cobas Fara centrifugal analyzer (Roche Diagnostics, Welwyn Garden City, U.K). Bradford reagent was prepared by dissolving 100mg of Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol. To this solution, 100ml 85% (w/v) phosphoric acid was added and stirred for 30 minutes before being diluted to 1000ml with distilled water and filtered through grade 1 Whatman filter paper.

Bradford reagent (256µl) was added to each cuvette and incubated for 100 seconds at 37°C, with an initial absorbance reading (595 nm) at 95 seconds. After the addition of 25µl of sample and 50µl distilled water, the cuvettes were incubated at 37°C for 180 seconds when a final absorbance (595 nm) was taken.

These absorbance values were read against a standard curve constructed using diluted BSA (0 to 100 mg/L). Samples over 100 mg/L were diluted with distilled water until they fell within the range of the standard curve.

2.037 Isolation and Culture of Thyrocytes

Feline thyrocytes were isolated from thyroid glands following thyroidectomy for the treatment of the disease ("adenomatous hyperplasia", "thyroid adenomas" "feline nodular goitre", referred to throughout the text as 'FNG'), or from cats immediately following euthanasia for diseases other than hyperthyroidism ('normal' thyrocytes referred to throughout the text as 'FT'). Thyroid glands from the Edinburgh area were refrigerated in 0.9% saline until collected (never more than 2 hours after removal). Glands from more distant sites were placed in RPMI media containing penicillin (100U/ml), streptomycin (100µg/ml), and L-glutamine (2 mmol/L) and sent by first class mail.

On receipt, glands were finely minced with scissors in Earle's balanced salt solution (EBSS) and repeatedly washed until most of the red blood cells were removed. Thyroid tissue was digested at 37°C for 2 hours in a 50ml enzyme cocktail of dispase (0.5% w/v), trypsin (0.25% w/v), collagenase (0.1% w/v) and BSA (2% w/v) in EBSS. The digest mix was shaken every 10 to 15 minutes during this period. Following digestion, the cell suspension was filtered through a 100µm

mesh gauze to remove undigested tissue. The resulting suspension was centrifuged at 125g for 5 minutes to pellet cells. The pellet was resuspended in RPMI media containing penicillin (100U/ml), streptomycin (100µg/ml), L-glutamine (2mmol/L), and 5% normal calf serum, filtered through a 30µm mesh gauze and centrifuged again to pellet cells. The pellet was then resuspended in RPMI media, the cells counted in a haemocytometer and plated into wells or flasks at a density of approximately $10x10^6$ cells per 75 cm^2 flask.

Cells were incubated at 37°C for 24 hours before being washed with EBSS to remove excess RBCs and fresh medium was then added (Figure 2.01).

2.038 Maintenance of FRTL-5 Cell Line

These TSH dependent, transformed rat thyroid follicular carcinoma cells were maintained in Coon's modified Ham's F-12 medium supplemented with a six hormone mixture consisting of bTSH (10mU/ml), bovine insulin (10µg/ml), hydrocortisone (10^{-8}M), transferrin (5µg/ml), somatostatin (10ng/ml), glycyl-L-histidyl-L-lysine acetate (GHL) (10ng/ml) and 5% newborn calf serum. They were passaged weekly with trypsin/EDTA(1x). Before growth or cAMP assays were carried out, the cells were deprived of TSH for 5 days to 'upregulate' the TSH receptor.

2.039 Maintenance of Chinese Hamster Ovary (CHO) Cells Tranfected With Human TSH (hTSH) Receptor (JPO9) and Untransfected (JPO2) CHO Cells.

These cells were maintained in Ham's F-12 medium and 5% newborn calf serum. They were passaged bi-weekly with 1x trypsin/EDTA(1x). They were tested and found to be mycoplasma free and re-tested every 6 months. When used for experiments, these cells were also supplemented with penicillin (100U/ml) and streptomycin (100µg/ml),

All cells (primary and cell lines) were cultured with the same batch of newborn calf serum to avoid batch variation between experiments.

2.040 IgG Preparation

Serum samples were treated with 6000MW PEG to a final concentration of 12.5% w/v to precipate immunoglobulins. Samples were then centrifuged at 1200g for 30 minutes at 4°C and the pellet redisolved in 20mM Na_2PO_4, pH 7.0. The resuspended immunoglobulins were then passed through a Staphlococcol Protein G column and the IgG subclass eluted in 0.1M glycine-HCl pH 2.7 according to the manufacturers recommendations.

Purity was checked by SDS-PAGE, demonstrating the presence of heavy and light chain bands (Figure 2.02). They were then concentrated in Amicon 10 microconcentrators by centrifugation at 12 000g. The samples were then dialysed against the medium used (NaCl free HBSS for cAMP assays, and medium for growth assays) and the protein concentrations in the final solution was determined via the Bradford method as described above. All samples were diluted to a final concentration of 10mg/ml and added to cells at a final concentration of 1mg/ml.

2.041 ^{3}H-Thymidine Incorporation For Cell Growth - Filter Method

All cell types used were plated at low density of 0.1 to 0.2 x 10^6 per ml. The following day, test substances were then added in fresh medium. For the last 6 hours of the experiment or overnight, 5μCi/ml [^{3}H]-TDr was added to each well. Following incorporation of [^{3}H]-TDr, the cells were gently washed twice with EBSS. Nonidet [1ml 0.1% (v/v)] in phosphate buffered saline (PBS) was then added to each well. Cells were incubated at 37°C for 30 minutes and the suspension removed from each well and the contents added to glass fibre filters attached to a vacuum manifold filtration unit. Wells were washed with a further 1ml 0.1% (v/v) Nonidet in PBS and the contents added to the respective filter. Each filter was then washed twice with 5ml of 0.1% (v/v) Nonidet in PBS. Ethanol (2ml) was added to each filter to facilitate drying and the filters dried in a fume cupboard. Once dry, the filters were placed in scintillation vials to which 3 ml scintillant was added. Scintillation vials were counted in a scintillation counter for 1 minute.

2.042 cAMP Radioimmunoassay

An 'in house' cAMP assay was developed using the following method.

i) Preparation of antibody reagents

0.1M Sodium acetate anhydrous (1.231g/150ml) was brought to a pH of 4.8-5.0 by the addition of 0.1M acetic acid ([572µl] glacial acetic acid/100ml water). This was diluted 1:2 with distilled water. Bovine serum albumin (BSA) (0.1%) was added immediately before use.

The primary antibody used was the kind gift of Dr Brent Williams of the Department of Medicine, The University of Edinburgh, Western General Hospital. It was developed by the injection of the acetylated form of cAMP into rabbits. Primary antibody dilution was determined for each new batch of tracer by performing an antibody dilution curve to establish 50% binding and was generally between 1:16 000 and 1:30 000.

The secondary antibody was made by mixing 20ml of donkey anti-rabbit serum with 1.5ml of normal rabbit serum and gently rotating overnight at 4°C. The resultant mixture was centrifuged at 230g for 15 minutes and the pellet resuspended in 10ml of 0.05M phosphate buffer and made up to 50ml.

ii) Preparation of standards, samples, controls and tracer.

Controls (3.2nM and 1.6nM) were made by diluting 32µM stock cAMP in acetate buffer without BSA and stored at -20°C for up to 2 months. The standard curve was prepared in acidified media (10ml medium to 100µl 20% acetic acid) at the time of experiment and acetylated with the samples.

Tracer was iodinated within the Department of Clinical Biochemistry by Mrs Susan Anema. Tracer was diluted such that approximately 4000cpm was added to each tube.

Samples were immediately acidified with 5µl 20% acetic acid for every 500µl of medium and frozen at -20°C. Acetylation with 15µl per 500µl sample of 2:1, triethylamine:acetic anhydride was

carried out immediately before each assay, along with the standard curve. Acetylation was carried out to improve the sensitivity of the assay.

iii) Assay

The radioimmunoassay was performed as follows: 50µl of acetylated sample or standard was added into duplicate tubes with 100µl of tracer. 100µl of primary antibody was then added to each tube (except totals and non-specific binding [NSBs]), the tubes vortexed and the assay incubated at 4°C overnight.

Following overnight incubation, 100µl of secondary antibody was added to each tube (except totals), the tubes vortexed, and placed on a shaker at room temperature for 1 hour. 3ml of wash solution (containing a small amount of microcrystalline cellulose, 1.5ml of Brij and 1L of distilled water) was added to each tube and the tubes immediately centrifuged at 1000G for 30 minutes. The supernatant was decanted by tipping and counted in a gamma counter for 6 minutes. Standard curve and interpolation of results was carried out by the "RIACALC" system (Wallace).

2.043 Radioimmunoassay of Thyrotropin Receptor Antibodies

Thyrotropin receptor antibodies were assayed using a commercially available RIA kit, (RSR Ltd, Cardiff). The assay was performed according to the pack insert instructions. Briefly, 50µl of sample was pipetted into 4ml tubes with 50µl of reconstituted TSH receptors. Tubes were vortexed for 5 seconds and allowed to incubate for 15 minutes. 100µl of ^{125}I-labelled TSH was added to each tube and the tubes vortexed for 5 seconds and incubated at room temperature for 2 hours. Precipitating solution (2ml at 4°C) was then added to each tube (except total tubes) and the tubes vortexed and centrifuged at 1500g for 30 minutes at 4°C. The supernatant was decanted by tipping, and the tubes counted in a gamma counter for 5 minutes. Modification of the assay was necessary when purified IgG was used (Chapter 6). In this case, the eluted IgG was diluted 1:2 with TRABs negative human serum to correct for non-specific binding and matrix effects.

2.044 Assay For Phosphoinositols

The assay was performed as follows:

i) Solutions prepared:

1M formic acid: ammonium formate (formic acid as salt)/0.1M formic acid (63.06g/L ammonium formate in 0.1M formic acid). Stable for up to 1 week at 4°C.
10mM EDTA (93mg/25ml): 1:1 (v/v) suspension of anion exchange resin in distilled H_2O.
500ml EBBS + 1g BSA + 0.5g glucose.
500ml EBBS + 1g BSA + 0.5g glucose + 10mM myo-inositol + 10mM LiCl.

ii) Experimental procedure

The inositol phospholipids are present in all mammalian cells. The procedure describes the radiolabelling of these lipids in response to cell stimulation. Cells are incubated in medium with [^{3}H]-inositol which becomes incorporated into inositol lipids through basal turnover.

Cells were preincubated with 10μCi/ml [^{3}H]-inositol for 48 hours in Ham's F-10 medium (low inositol medium). The medium was then removed from each well and replaced with 0.5ml of EBS/BSA (0.2%)/glucose (0.1%) and incubated for 15 minutes to wash away the extracellular tritiated inositol. This medium was removed from each well and replaced with 0.45ml of EBS/BSA (0.2%)/glucose (0.1%) with 10mM cold inositol for a further 15 minutes to allow the unlabelled inositol to enter the cells and displace the tritiated inositol, and also for the lithium to inhibit the inositol phosphate phosphatases. At the end of the 15 minute incubation period test substances were added as required to triplicate wells in a 50μl volume. Cells were then incubated as required (usually for two hours).

The experiment was terminated by the addition of 250μl of ice cold 15% perchloric acid. All of the well contents were removed to a 1.5ml Eppendorf tube. The wells were rinsed with 0.5ml water and transfered to the same Eppendorf tube. Samples were spun in a microfuge at 3300g for 3 minutes. The supernatant (containing phosphoinositols) was removed to a glass tube and processed as described subsequently.

iii) Supernatant processing : recovery of inositol / phosphoinositols

1. 1.5 ml of trichlorotrifluroethane and octylamine (1:1v/v) were added to each sample and mixed on a vortex mixer.
2. Samples were then centrifuged at 1 500g for 2 to3 minutes on a bench microcentrifuge to separate into 3 phases.
3. 0.9 ml of aqueous upper phase was removed for processing as stated subsequently, taking care not to remove the lower phase.

iv) Assay for inositol / phosphoinositols

1. 100µl EDTA was added to each sample to chelate calcium.
2. 0.5ml resin/water mix was added to each column. 4ml distilled H_20 was added to each to rinse the column and prevent air locks.
3. Samples were loaded onto each column. Samples were then rinsed with 1 ml dH_20 plus 2x4ml of dH_20 and allowed to drain completely to remove unbound inositol.
4. Over a rack of scintillation vials, 2ml ammonium formate/formic acid buffer was added to each column to elute radiolabelled inositol phosphates into the scintillation vials. The procedure was repeated once.
5. 3ml scintillation fluid was added to each vial and the tubes counted in a scintillation counter.

iv) Calculation of results

The results were calculated by adding the disintigrations per minute (DPM) obtained from the two column fractions. As only 0.9ml of the total cell extract (1.25ml) was used, a factor (of 1.4) was used to correct for the dilution of the sample.

2.045 Radioimmunoassay of Thyroid Hormones

The TT4 (DPC; TT4) and TT3 (Amerlex; MT3) assays were optimised and validated for use with cat serum, in advance of assaying the samples, by SCL Diagnostics as described in detail in Chapter 9.02. Serum reverse T3 concentrations were determined by radioimmunoassay using a primary antibody with polyethylene glycol separation system (Biostat Diagnostics).

2.046 Cloning of the Feline TSH Receptor

i) DNA extraction and polymerase chain reaction (PCR)

Genomic DNA was obtained from thyroid and uterine tissue and peripheral blood leukocytes using SDS lysis, proteinase K digestion and phenolchlorophorm extraction. Oligonucleotide primers based on areas of homology between exon 10 of the human, bovine and canine TSHR gene were designed to yeild a 537 base pair (bp) PCR product encompassing codons 480 to 640 of the feline TSH receptor. The sequence of these primers was FeTSHR10F 5' CTG GTA GAC CTC TAC ACT CAC TCT GAG 3" and FeTSHR10R 5' GTT CAG AAT TGC TGA CAG AGC ATA 3". PCR was performed at an annealing temperature of 60°C.

ii) Single stranded conformational polymorphism analysis and DNA sequencing

The feline TSHR PCR product was cleaved with the restriction enzyme NcoI (NBL Gene Sciences, Cramlington, UK) to yield digestion products of 302 and 235 bp. Single stranded conformational polymorphisms (SSCPs) were resolved on precast 12.5% polyacrylamide minigel, which had been precooled to either 10°C or 20°C and the gels (Phast system, Pharmacia LKB, Uppsala) were run for 240 Vhrs. Samples were each run in duplicate at both temperatures on different days. The DNA sequences of both strands of PCR products were determined by semiautomated cycle sequencing (Applied Biosystems, 377 sequencer, Foster City, CA) as described. The normal sequence of the feline TSHR from codon 480 to 640 was determined by analysis of DNA from normal feline hysterectomy specimens and compared with that of the thyroid DNA from five thyrotoxic cats and peripheral blood DNA from two thyrotoxic littermates using Sequence Navigator software (Applied Biosystems).

2.047 Plasma Selenium Concentrations

The method of detecting selenium in serum samples is based on the formation of a piazselenol between selenium and 2,3-diaminonaphthalene followed by extraction and flourometry.

Plasma samples were stored at -80°C until analysis was carried out. The assay has been described elswhere (Olsen *et al.*, 1975). Briefly, 0.25ml plasma was digested overnight in 2ml

concentrated nitric acid (BDH) in a 75ml "quickfit" glass boiling tube. The samples were then heated slowly to boiling point and kept boiling until brown fumes disappeared (approximately 5 minutes). Concentrated perchloric acid (2ml) (BDH) was then added dropwise to the sample and the samples then boiled until white fumes appeared. Boiling was continued for 30 minutes when 2ml 10% (v/v) hydrochloric acid (BDH) was added dropwise to the samples to drive off any excess nitric acid and to convert selenium as selenate to selenite.

When cooled, 5ml hydroxyamine/EDTA solution (25g hydroxylamine and 9.24g EDTA per litre distilled water) was added to each sample, followed by five drops of the colour indicator (cresol red [0.05g cresol red in 250ml distilled water containing 1ml of 40% {v/v} ammonia]). Ammonia solution (40% v/v in distilled water) (BDH) was added to each sample until the sample turned green (approximately 3ml), then 10% (v/v) HCl was added to each sample to turn the colour of the solution orange (approximately 2ml). This brought the pH of the sample to 1.5 to 2.5 which is ideal for the formation of the diaminonaphthaline-selenium complex. The samples were then diluted to 50ml with distilled water.

Diaminonaphthalene solution (5ml) was added to each sample and incubated for 30 minutes at 50°C in a covered water bath. The samples were then cooled to room temperature and 6ml cyclohexane (Analar, BDH) was added to each sample. Each tube was covered with a glass stopper and shaken vigorously for 20 seconds to extract the diaminonaphthalene/selenium complex. The sample was left for 10 minutes to allow the diaminonapthalene layer to separate. 2.5ml of the top layer was removed and the fluorescence was measured. The samples were read off a standard curve, prepared from selenous acid (BDH) supplied at 1mg/ml. Standards were: 0µg/ml, 25µg/ml, 50µg/ml and 100µg/ml. A dried blood standard was used as a control (Analytical Quality Control Services, Atomic Energy Authority, Austria).

2.048 Glutathione Peroxidase Activity Assay

Initially, a reaction mix was made from 5mg $NADPH_2$, 46mg reduced glutathione, 3ml distilled water, 24ml PBS, 1ml sodium azide (0.1125M), and 20U glutathione reductase. A 0.0022M solution of H_2O_2 was also made prior to the beginning of this assay.

The following method was used to detect GPX activity in red blood cells (RBC-GPX) and plasma (P-GPX). Whole blood samples were diluted 4 fold with PBS and mixed gently until no clots were present. Plasma samples were diluted 1 in 10 with PBS. Red blood cells (in whole blood

samples) were lysed by dilution (1:20) in distilled water. Plasma samples were not further diluted. To measure the GPX activity in samples, 955µl of reaction mix, 10µl sample and 35µl H_2O_2 was added to a cuvette. The rate of change of absorbance was followed at 340nM. A rate for a reacgent blank (reaction mix plus H_2O_2) was subtracted from the rate in the samples.

Results were calculated as follows:

A unit of glutathione peroxidase is defined as that which oxidises 1µM of NADPH per minute. The molar extinction coefficient of NADPH is 6220. The conversion factor for the assay was 16.077 and was calculated as follows:

$$\frac{x}{6220} \times \frac{1}{1000} \times \frac{1\times10^{6}}{1} \times \frac{1000}{10}$$

molar extinction coefficient | correction to 1L | for µM | amount of lysate used

x dilution factor of original sample (20 for RBCs, 10 for serum).

Haemoglobin (Hb) concentrations were determined by the addition of 1µl of sample with 250µl Drabkin's reagent and incubated for 15 minutes. The absorbance was read from an automated plate reader (Dyrex Laboratories MRX, U.K.) with 18g/dl Hb as standard.

2.049 Statistical Analysis

The specific tests used to statistically analyse results are described in the Materials and Methods section of each results chapter. Tests were chosen under the direction of staff from the Department of Medical Statistics and Computing, The University of Edinburgh. All tests were considered significant if they had a p value of < 0.05. All statistics were performed using Minitab Release 10xtra for the Power Macintosh.

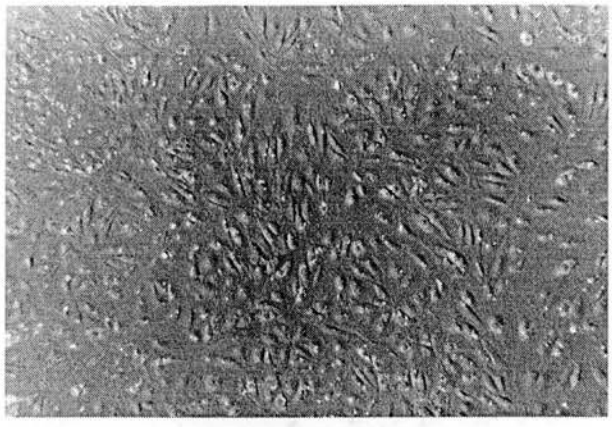


Figure 2.01a: Feline thyrocytes in culture at 2 days (x 20 magnification).

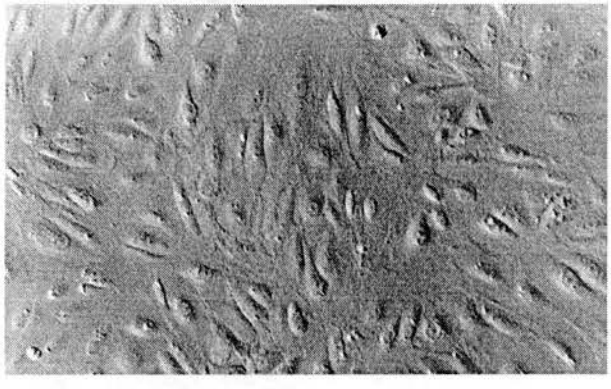


Figure 2.01b: Feline thyrocytes in culture at 2 days (x40) magnification.

Figure 2.01: Photograph of normal feline thyrocytes in culture at 2 days.

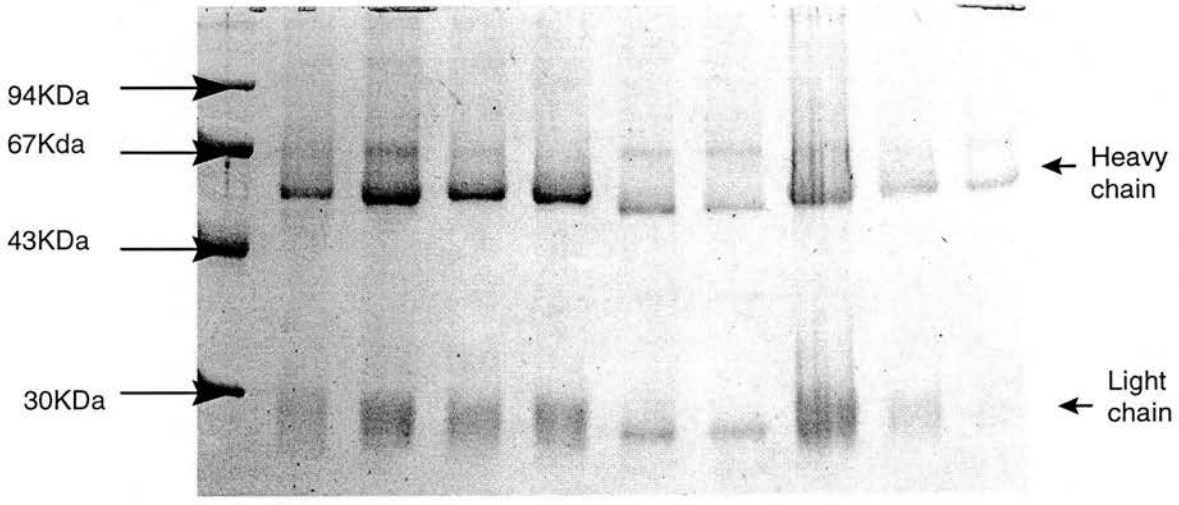


Figure 2.02: SDS-PAGE of nine feline immunoglobulin G (IgG) preparations illustrating heavy and light chain bands.

3.00 THYROID HORMONE DEIODINATION IN THE DOMESTIC CAT

3.01 INTRODUCTION

The thyroid gland provides the sole source of circulating T4 which is regarded as a prohormone requiring 5' monodeiodination to produce the metabolically active hormone T3. In selenium replete humans and rats, approximately 20 per cent of circulating T3 originates from the thyroid gland, whilst 80 per cent of T3 in plasma appears to arise from 5'-mono-deiodination of T4 in a number of tissues, especially the liver and kidney (Silva and Larsen, 1986; Kohrle, 1994; St Germain, 1994). Deiodination of thyroid hormones plays a crucial role in the regulation of T3 supply to both tissue and blood.

Deiodination of thyroxine and other iodothyronines is catalysed by the family of iodothyronine deiodinases (ID) and current evidence suggests that these enzymes provide an autoregulatory role in many tissues to maintain intracellular T3 in response to altered thyroidal secretion of thyroid hormones. Three selenoenzymes are involved in the deiodination of thyroid hormones, namely iodothyronine deiodinase types I, II and III (IDI, IDII, IDIII). IDI is the major isoenzyme found in liver and kidney and this enzyme appears to provide the major proportion of T3 found in plasma (St Germain and Croteau, 1989). IDI is also expressed in high concentrations in the thyroid of some species including humans, dogs and rodents but many animal species including goats, cattle and sheep fail to express the enzyme, or at least express it at very low levels within the gland (Beckett, Beech, Nicol *et al.*, 1993). IDI can catalyse both 5' and 5 mono-deiodination to produce T3 and rT3, respectively, from T4. In rats and humans, IDI appears to show a substrate preference for rT3, although it can readily act on T4 (Visser, Kaptein, Terpstra *et al.*, 1988).

A number of methods have been employed to assay for IDI expression and activity. Expression of the enzyme is usually assessed with an affinity labelling technique using ^{125}I-bromoacetyl

derivatives of rT3, T3 or T4 (Kohrle, Rasmussen, Ekenbarger *et al.*, 1990; Schoenmakers, Pigmans and Visser, 1992). Activity is measured by assessing the ability of IDI and IDII to metabolise T4 or rT3 in the presence of an active thiol agent such as dithiothreitol (DTT). IDI and IDII show very different sensitivities to inhibition by the addition of propylthiouracil (PTU) and aurothioglucose (AuG) (St Germain, 1994). IDI activity with rT3 or T4 as substrate is readily inhibited by both these agents, whilst IDII is relatively resistant to inhibition by PTU or AuG . These inhibitors are thus often used to indicate which isoenzyme is expressed by tissues.

Previous studies have suggested that IDI has similar properties in all species investigated. The main differences reported have been confined to small variations in molecular mass (Schoenmakers, Pigmans and Visser, 1992), differences in turnover number of approximately 10-fold between species (Santini, Chopra, Hurd *et al*, 1992; Schoenmakers, Pigmans and Visser, 1992) and differences in sensitivity to inhibition by AuG (Santini, Chopra, Hurd *et al*, 1992). However there have been reported clear species-differences concerning the degree of IDI expression by the thyroid (Beckett, Beech, Nicol *et al.*, 1993; Beech, Walker, Dorrance *et al.*, 1993).

The aims of this study were therefore to:

- examine the expression of IDI in the thyroid, liver and kidney of the domestic cat
- examine the sensitivity of IDI to PTU and AuG
- examine the kinetics of IDI to the substrates rT3 and T4

3.02 MATERIALS AND METHODS

a) ANIMAL TISSUES

Tissues (liver, kidney and thyroid) were obtained from cats not suffering from diseases of the relevant organs immediately following euthanasia, placed in liquid nitrogen and stored at -80°C until assays were carried out. Rat tissues were obtained and stored in a similar manner.

b) PREPARATION OF TISSUE HOMOGENATES AND MICROSOMAL FRACTIONS

Portions of tissues were homogenised in 100mMKH_2PO_4/1mMEDTA buffer pH 7.4 (IDI assay buffer), at 20% w/v, and processed as described in Chapter 2.031

c) PROTEIN DETERMINATIONS

Protein concentrations were determined by the method described by Bradford (1976) using BSA as standard as described in Chapter 2.036.

d) ASSAY OF DEIODINASE ACTIVITY USING ^{125}I-rT3 AS SUBSTRATE

The assay procedure was based on that of Sawada *et al.* (1986). Prior to use, 200µl of ^{125}I-rT3 (9250KBq/1.25ml; 27.8- 46.3MBq/µg) was purified as follows. Free ^{125}I was removed by passing the tracer down a 2ml Sephadex G25 column in 100mmol/l potassium phosphate/1mmol/l EDTA (pH 7.4) (potassium phosphate buffer). The column was washed with 20ml of potassium phosphate buffer to remove contaminating ^{125}I and the ^{125}I-rT3 was eluted with a 1% (w/v) solution of BSA prepared in IDI assay buffer.

Tissue homogenates (n=5) (post-1500g centrifugation) (200µl) in duplicate, were incubated with 100µl of purified ^{125}I-rT3 at a final activity of 0.0185MBq/ml in IDI assay buffer in the presence of 20mmol/l DTT. Unlabelled rT3 was added at a final concentration of 5µM. For kinetic experiments, rT3 was added at concentrations spanning the range of 5 to 500µM. Samples were incubated at 37°C for the times indicated in the results section and the reaction was stopped by the addition of 0.4mls of normal human sera followed by 0.4ml 40% TCA. After centrifugation at 1500g for 10 minutes, 0.8ml supernatant was removed and counted together with tubes containing the remaining supernatant and pellet. The percentage of ^{125}I released was calculated and used to determine the mass of rT3 that had been metabolised (as described in Chapter 2.031).

e) EFFECTS OF PTU AND AuG ON DEIODINASE ACTIVITY.

The inhibition of IDI in assays for activity was investigated by the addition of 1mM PTU or 12nM AuG (final concentration). These conditions were chosen as these concentrations of PTU and AuG are known to inhibit IDI activity in other species (Leonard and Rosenberg, 1978; Visser and Overmeeren-Kaptein, 1981; Goswami and Rosenberg, 1986; St Germain and Croteau, 1989; Berry, Kieffer, Harney *et al.*, 1993)

f) ASSAY OF IDI ACTIVITY IN TISSUE HOMOGENATES USING T4 AS SUBSTRATE

This was performed as described previously (Beckett, Beddows, Morrice *et al.*, 1987) and in Chapter 2.032. Briefly, a solution of T4 (56µM) was prepared in 0.25M sucrose/0.05M Tris/1mM EDTA buffer pH 7.4 containing 3% BSA. Tissue homogenates (2ml) were then preincubated for 10 minutes at 37°C and T4 (200µl)(0.5 to 510nM final concentration) was added to start the reaction. At designated time points, 200µl of sample was removed in duplicate into 400µl of ethanol and centrifuged at 1500g for 5 minutes. The concentration of T3 was then determined by immunoassay using a Vitros Eci immunoassay analyser (Ortho Clinical Diagnostics, Rochester, U.S.A).

g) AFFINITY LABELLING OF IDI IN MICROSOMAL LIVER FRACTION

N-Bromoacetyl-^{125}I-rT3 (BrAc ^{125}I-rT3) was prepared as previously described (Beckett, Beddows, Maurice *et al.*, 1987) and in Chapter 2.033. Microsomal fractions isolated from tissues as described (Chapter 2.031) were diluted to a final concentration of 1mg/ml protein in 50mM TrisHCl, 3mM EDTA and 3mM DTT. Microsomes (50µl, 50µg protein) were then added to 0.02MBq of N-Bromoacetyl-[^{125}I]rT$_3$ affinity label, which had been evaporated to dryness under dry nitrogen. The reaction was allowed to proceed for 15 minutes.

For experiments designed to study the effects of PTU and AuG on IDI affinity labelling, either 1mM PTU or 40nM AuG was added to each test prior to addition of the affinity label. The effects of rT3 and PTU on affinity labelling were also studied with the addition of 6nM rT3 together with 1mM PTU.

After labelling, samples were diluted 2:1 with 'boiling mix' consisting of SDS (35mmol/L), glycerol (1.4mmol/L), 2-mercaptoethanol (0.3mmol/L) and bromophenol blue (15mmol/L) and then heated for 10 minutes at 90°C. Samples were subjected to SDS-PAGE using a 12% (w/v) gel, and the ^{125}I-labelled proteins visualised using autoradiography.

h) DETERMINATION OF DEIODINATION KINETICS OF rT3 AND T4 FOR IDI

Km and Vmax values were determined according to the Michaelis-Menten equation by construction of Lineweaver-Burk plots (Figure 3.01). Final concentrations of rT3 and T4 ranged from 100nM to 50µM and 0.5nM to 510nM respectively.

i) SERUM rT3 CONCENTRATIONS

A commercial rT3 RIA kit (Biostat Diagnostics, Stockport, UK) was used to measure the rT3 concentrations in serum from 10 euthyroid cats and 10 euthyroid rats.

j) STATISTICAL ANALYSIS

The Mann Whitney U test was used to test for significant difference between the rT3 concentrations in cats and rats.

3.03 RESULTS

a) IODOTHYRONINE DEIODINASE ACTIVITY IN TISSUE HOMOGENATES AND MICROSOMAL FRACTIONS USING rT3 AND T4 AS SUBSTRATES

i) ^{125}I-rT3 as substrate

When ^{125}I-rT3 (6nM) was used as substrate, feline liver and kidney homogenates showed approximately 0.2% of the deiodinase activity found in rat liver (Table 3.01). Because of the low deiodinase activity found in feline liver and kidney, it was necessary to use an incubation period of between 4 and 24 hours whilst in contrast, using rat liver, an incubation period of only 5 minutes with a 1:10 dilution of homogenate was required. In feline liver and kidney homogenates, deiodination was found to proceed at a linear rate (Figs. 3.02). No deiodinase activity could be found in thyroid homogenates from three euthyroid and five thyrotoxic cats, even when the incubation periods were as long as 48 hours (Table 3.01). Propylthiouracil (1mM) and AuG (12nM) completely inhibited the deiodinase activity seen with 20mM DTT in both rat liver homogenates and caused a reduction in activity to approximately 0.2% of the basal activity in cat liver (Table 3.01).

ii) T4 as substrate

When T4 was used as substrate, the deiodinase activity in rat and feline liver were similar (Table 3.01). PTU and AuG markedly inhibited deiodination in cat and rat liver such that less than 10% of the initial activity remained.

b) KINETIC STUDIES OF RAT AND FELINE IDI

The kinetic data for rat and feline liver homogenates for rT3 and T4 are shown in Table 3.02. Lineweaver-Burk plots are illustrated for rat liver (n = 5) (Fig. 3.03) and cat liver (n = 5) (Fig.

3.04). The Km for rat and cat liver using rT3 as substrate were markedly different, cat liver being approximately five fold greater than that for rat liver. However, the Km values using rT3 in cat liver homogenates could only be approximated as it was impossible to obtain rT3 in solution at concentrations greater than 50μM. Thus data points with substrate concentrations well above the Km of the enzyme could not be used.

With T4 as substrate, the Km and Vmax of the enzymes in rat and feline liver homogenates were very similar. Lineweaver-Burk plots are illustrated for rat liver (n = 5) (Fig. 3.05) and cat liver (n = 5) (Fig. 3.06).

c) BROMOACETYL rT3 AFFINITY LABELLING

In rat liver microsomes (n = 4), affinity labelling with ^{125}I-rT$_3$-BrAcrT3 demonstrated an ^{125}I -labelled band with a Mr of 29.1±0.94 KDa. This labelled protein has previously been shown to be IDI in this tissue and was used as a positive control on all gels. The labelling of this protein was prevented in the presence of PTU and AuG (Figs. 3.07 to 3.14). Similarly, in feline liver and kidney microsomes, a ^{125}I-labelled band was found with Mr 26.23±0.33 KDa and 27.16±0.12 KDa (Figs. 3.07 & 3.09) respectively. As in rat liver, the labelling of this protein was prevented by the addition of PTU (Fig. 3.07 for feline liver & Fig.3.09 for feline kidney) and AuG (Fig. 3.08 for feline liver & Fig. 3.10 for feline kidney). The PTU and AuG inhibited affinity-labelled (27 KDa) protein in cat liver and cat kidney, showed a similar labelling intensity to that of the 29kD labelled protein found in rat liver. Although there were affinity-labelled proteins in feline thyroid and cerebral cortex microsomes, none were inhibited by the addition of PTU (Fig. 3.11 for feline thyroid & Fig. 3.13 for feline cerebral cortex) or AuG (Fig. 3.12 for feline thyroid & Fig. 3.14 for feline cerebral cortex).

d) SERUM rT3 CONCENTRATION IN EUTHYROID CATS

The median serum rT3 concentration in cats was 0.164 nmol/l (range 0.086 to 0.244 nmol/l; Q1: 0.099 nmol/l; Q3: 0.204 nmol/l). The median serum rT3 concentration in rats was 0.3305 nmol/l range 0.2670 to 0.3710 nmol/l; Q1: 0.2868 nmol/l; Q3: 0.3593 nmol/l). The serum rT3 concentration in cats was significantly lower than in rats (p = 0.0010).

Table 3.01: IDI Activity In Tissue Homogenates (n = 5) With rT3 (5M) and T4 (50nM) as Substrates

Substrate	Median		Range	Q1	Q3	
rT3 (deiodinase activity in fmol/mg/min)						
Rat liver	23800	22855	-	25622	22885	28111
Rat liver plus PTU (1mM)	ND					
Rat liver plus AuG (12nM)	ND					
Cat liver	40.85	24.84	-	62.05	27.44	57.97
Cat liver plus PTU (1mM)	0.07	0	-	0.10	0.03	0.85
Cat liver plus AuG (12nM)	0.05	0	-	0.09	0.02	0.82
Cat kidney	68.79	63.22	-	71.04	64.01	66.54
Cat thyroid	ND					
T4 (deiodinase activity nmol/mg/min)						
Rat liver	3.71	3.51	-	3.91	3.56	3.86
Rat liver plus PTU (1mM)	0.382	0	-	0.412	0.02	0.39
Rat liver plus AuG (12nM)	0.02	0	-	0.05	0	0.04
Cat liver	3.61	3.41	-	3.81	3.46	3.76
Cat liver plus PTU (1mM)	0.002	0	-	0.01	0.001	0.008
Cat liver plus AuG (12nM)	ND					

AuG, aurothioglucose; ND, not detectable; PTU, propylthiouracil; Q1, first quartile; Q3, third quartile

Table 3.02: Kinetic data for Rat and Feline Liver Homogenates (n = 5)

Species	Substrate	Median		Range		Q1	Q3
	rT3						
Rat							
Km (M)		0.20	0.16	-	0.40	0.18	0.30
Vmax(pmol/mg/min)		100.00	12.50	-	100.00	31.30	100.00
Cat							
Km (M)		3873.00	2053.00	-	7857.00	2240.00	7130.00
Vmax(pmol/mg/min)		56.20	25.00	-	142.80	27.00	132.10
	T4						
Rat							
Km (M)		2.25	0.54	-	3.06	0.54	3.06
Vmax(pmol/mg/min)		0.63	0.18	-	2.50	0.18	2.50
Cat							
Km (M)		1.53	0.44	-	4.20	0.44	4.20
Vmax(pmol/mg/min)		0.25	0.07	-	0.34	0.07	0.34

Q1, first quartile; Q3, third quartile

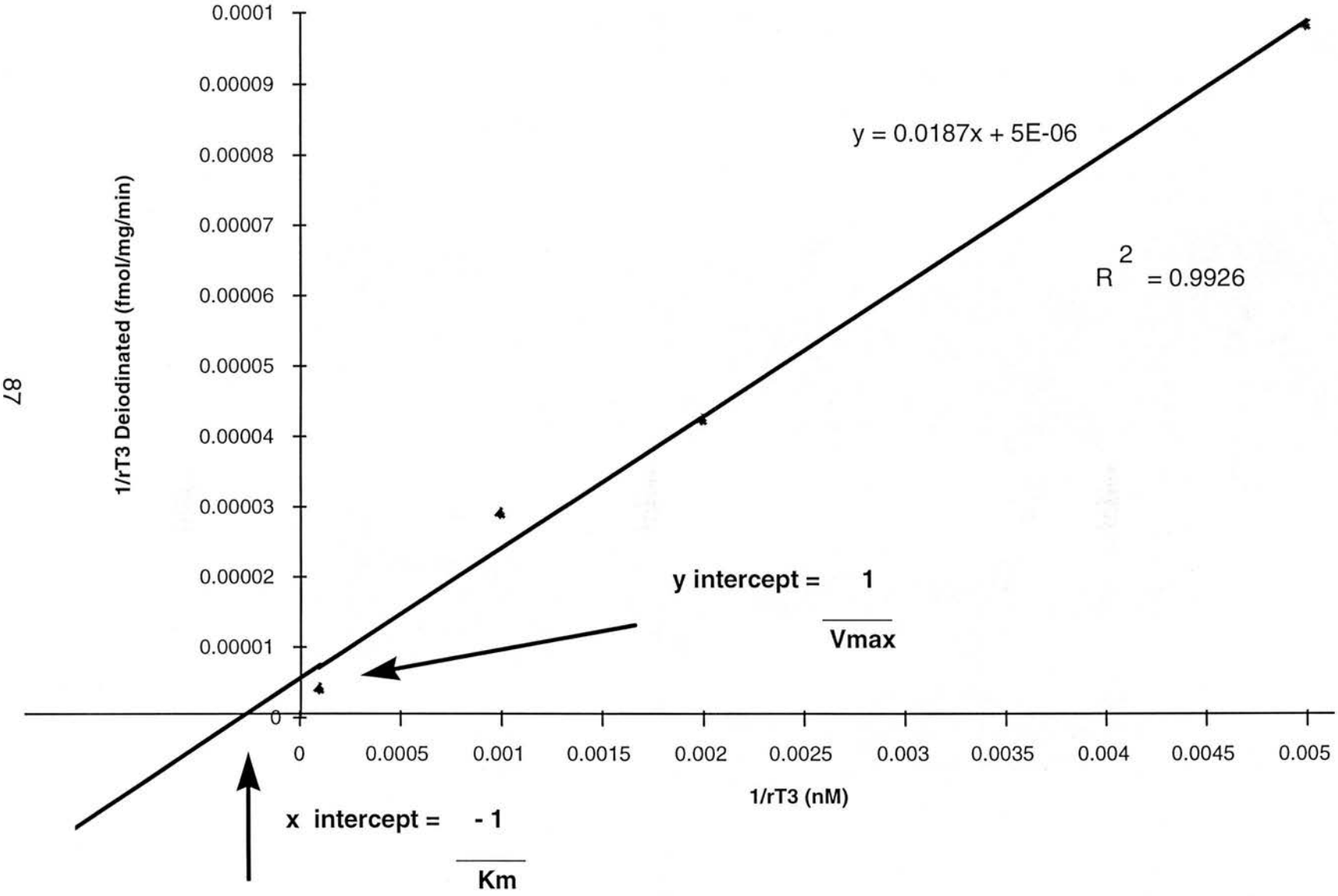


Figure 3.01: An example of Lineweaver-Burk reciprocal plot showing rT3 deiodination by rat liver homogenates.

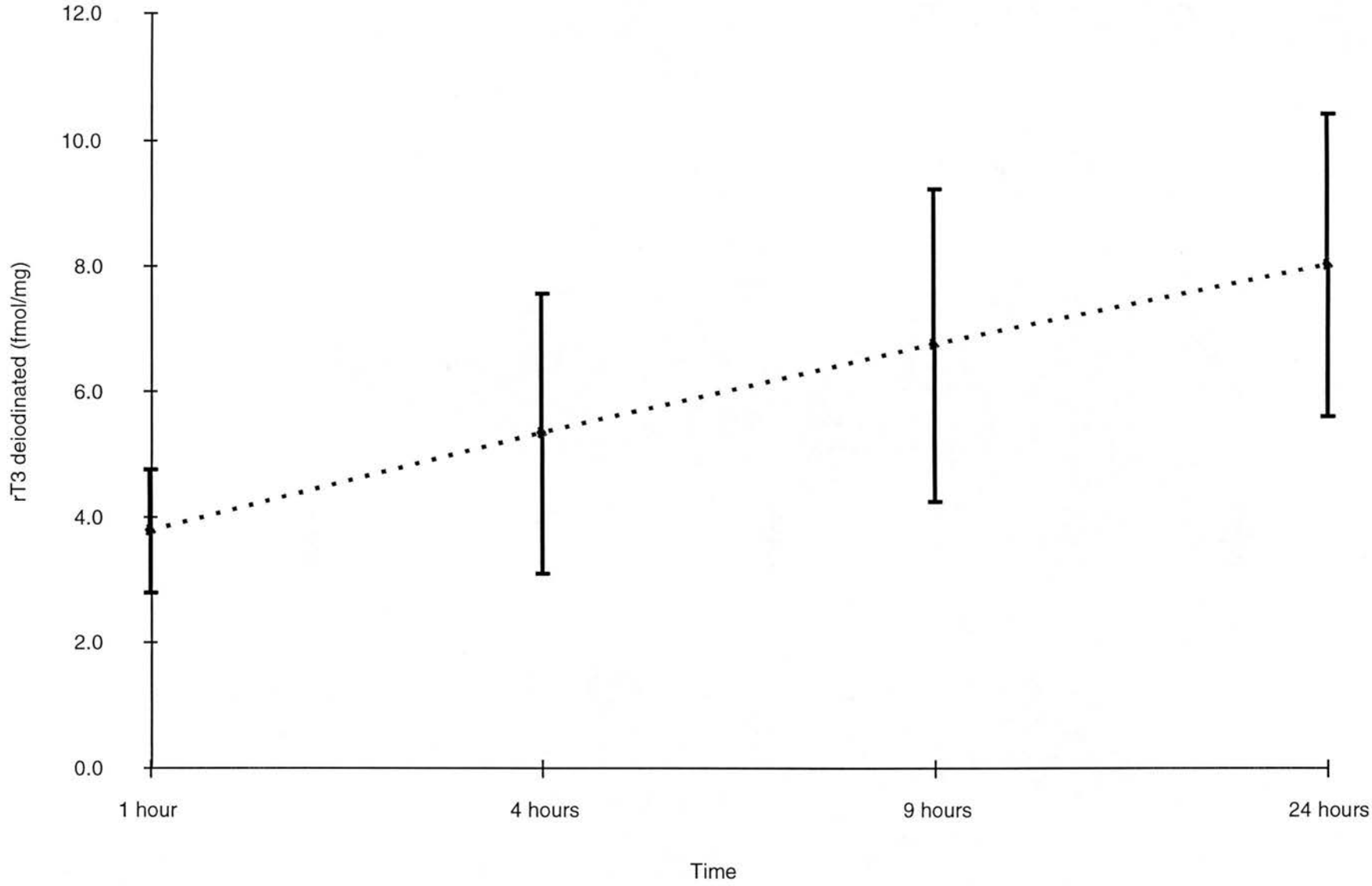


Figure 3.02: Deiodination of rT3 in 5 feline liver homogenates. Results shown are those of the mean ± SEM.

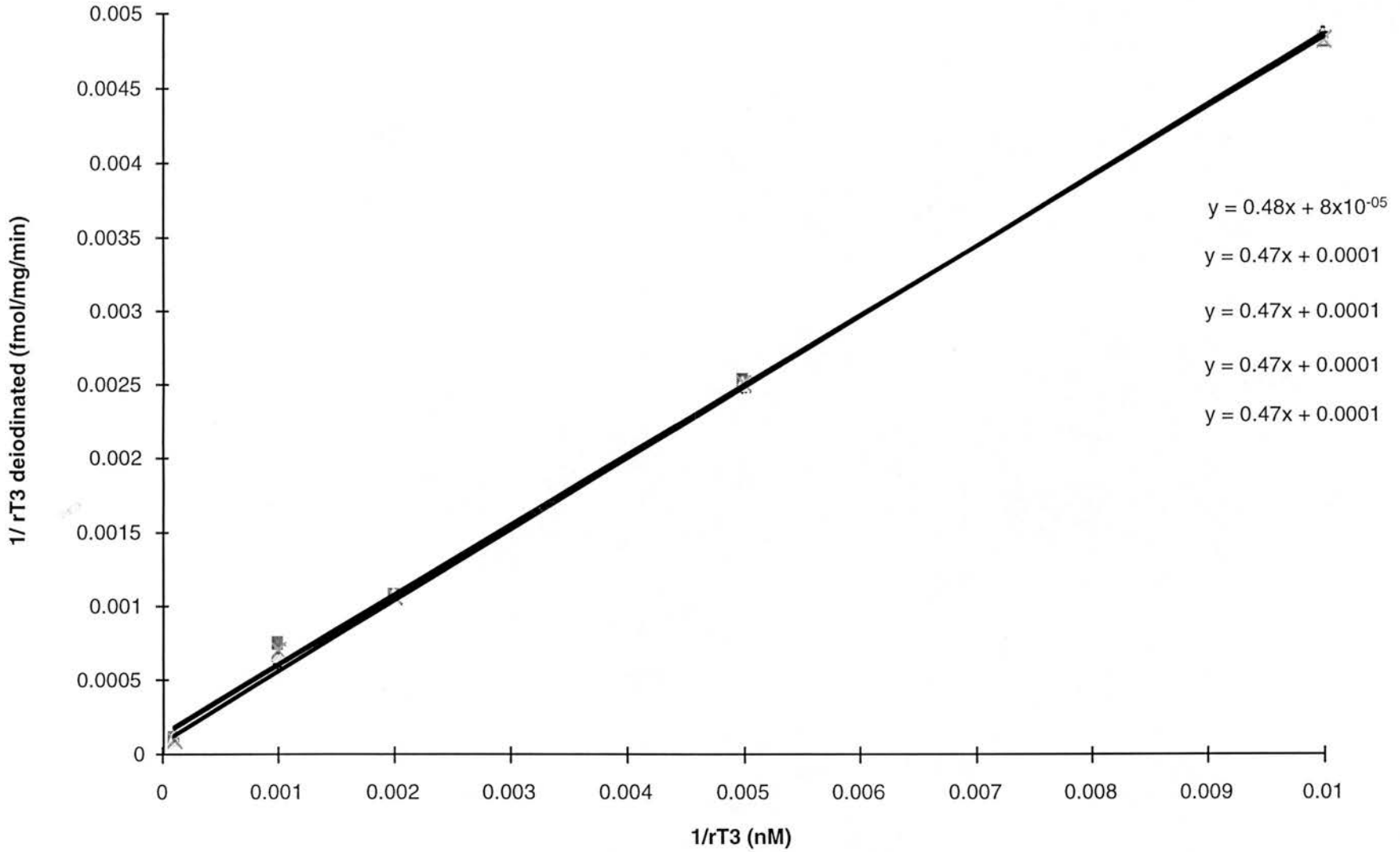


Figure 3.03: Lineweaver-Burk Plots showing rT3 deiodination by rat liver homogenates. Experiments using 5 different liver homogenates are shown.

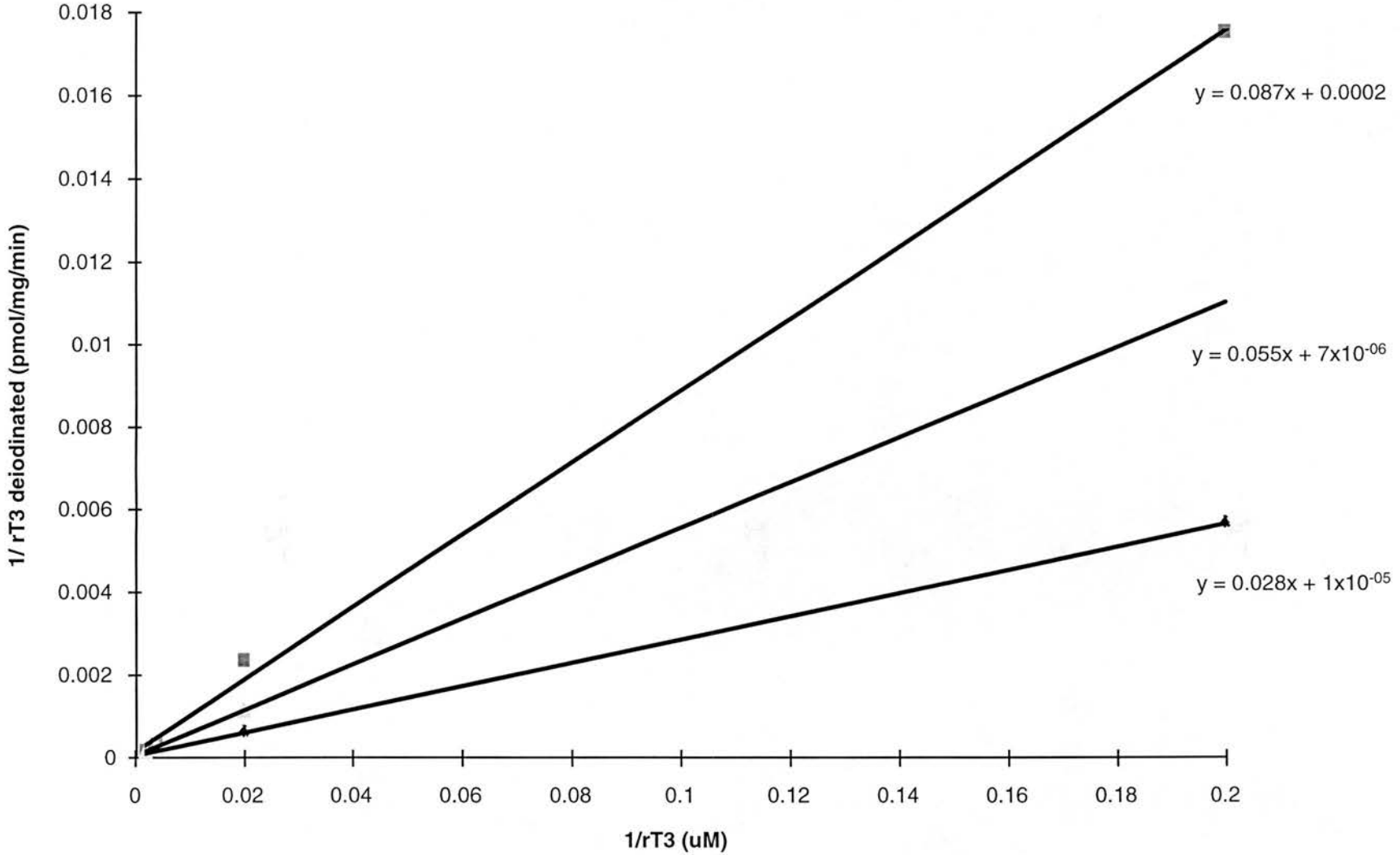


Figure 3.04: Lineweaver-Burk Plots showing rT3 deiodinated by feline liver homogenates. Experiments using 3 different liver homogenates are shown.

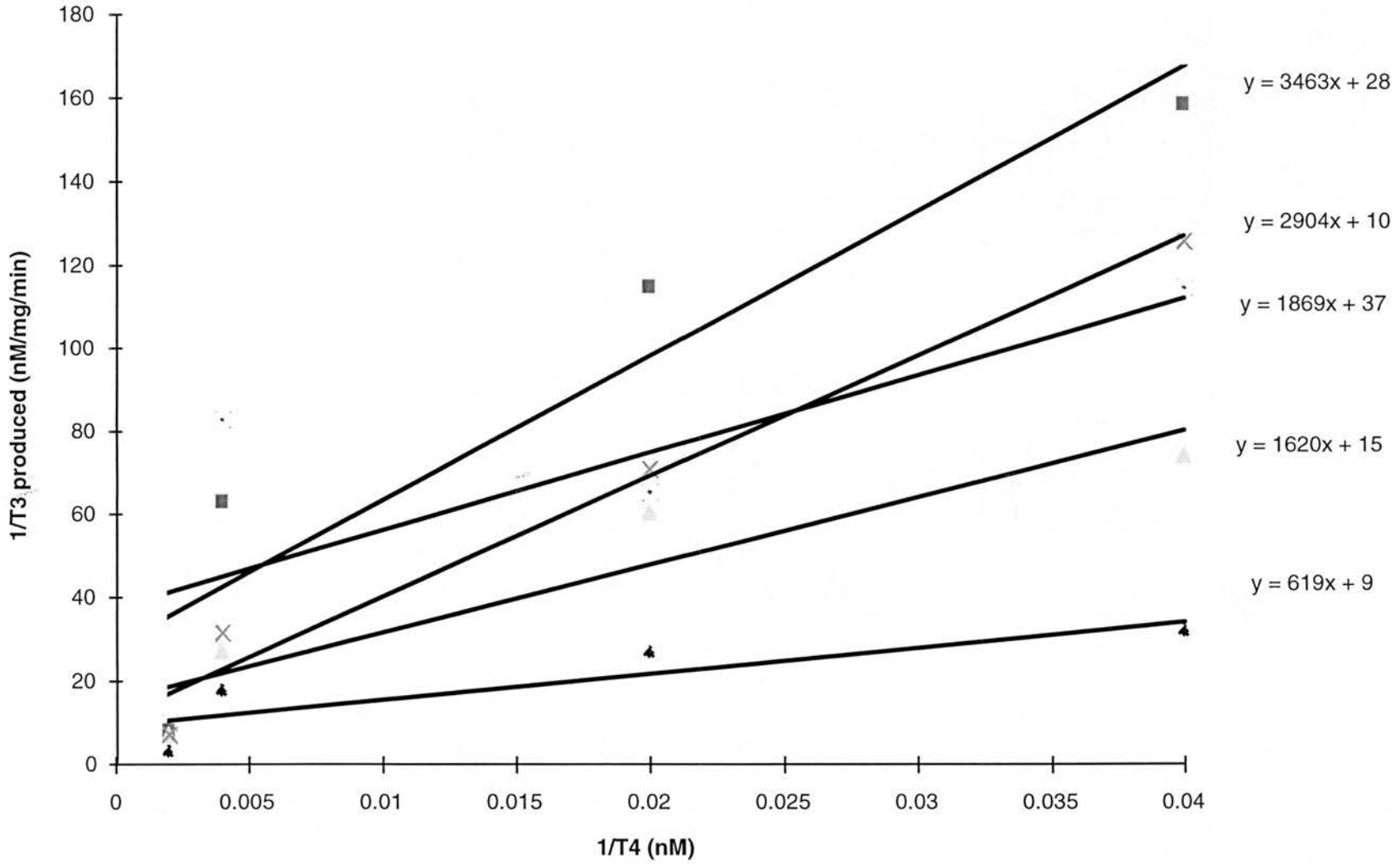


Figure 3.05: Lineweaver-Burk Plots showing T4 deiodination by rat liver homogenates. Experiments using 5 different liver homogentaes are shown.

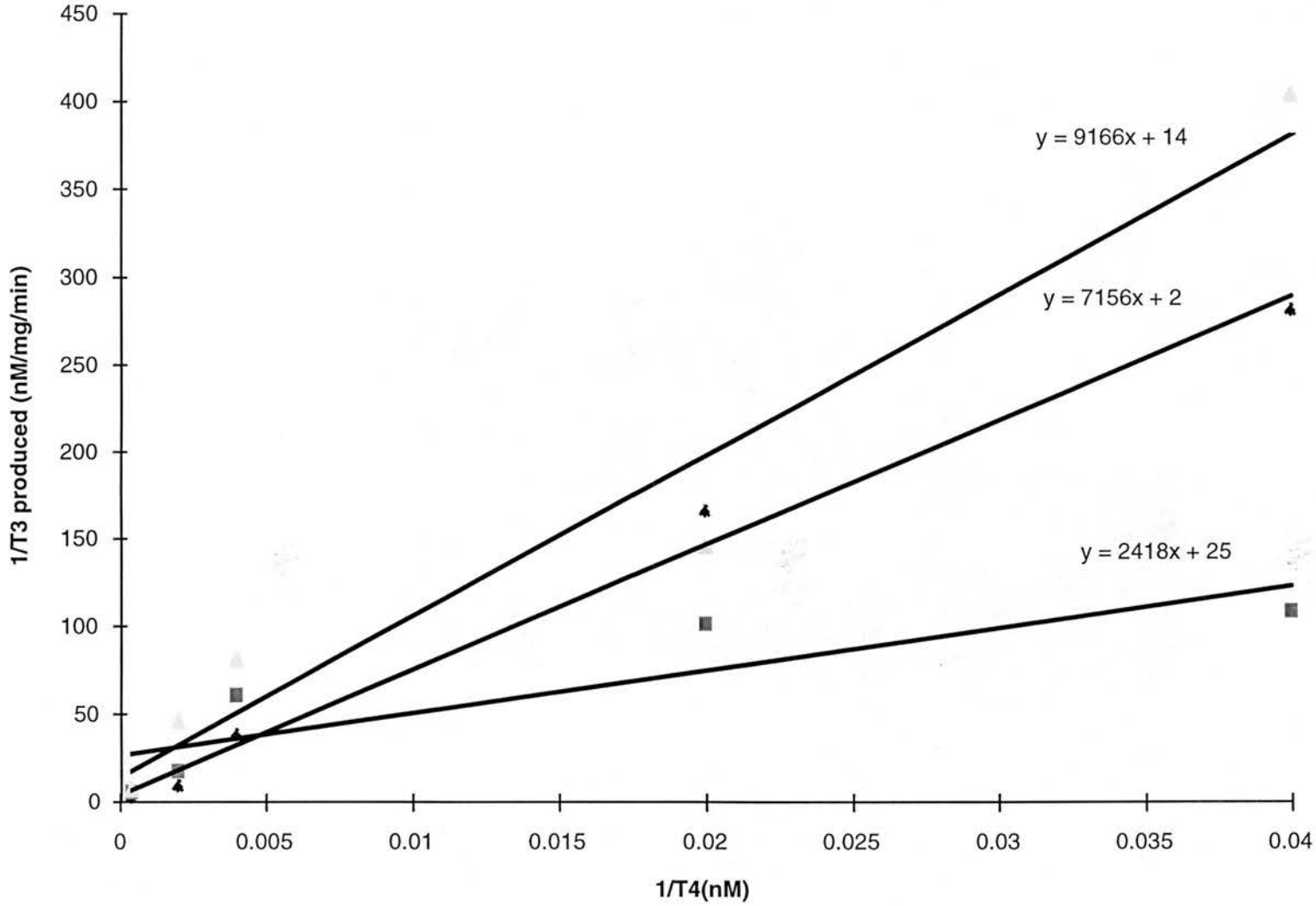
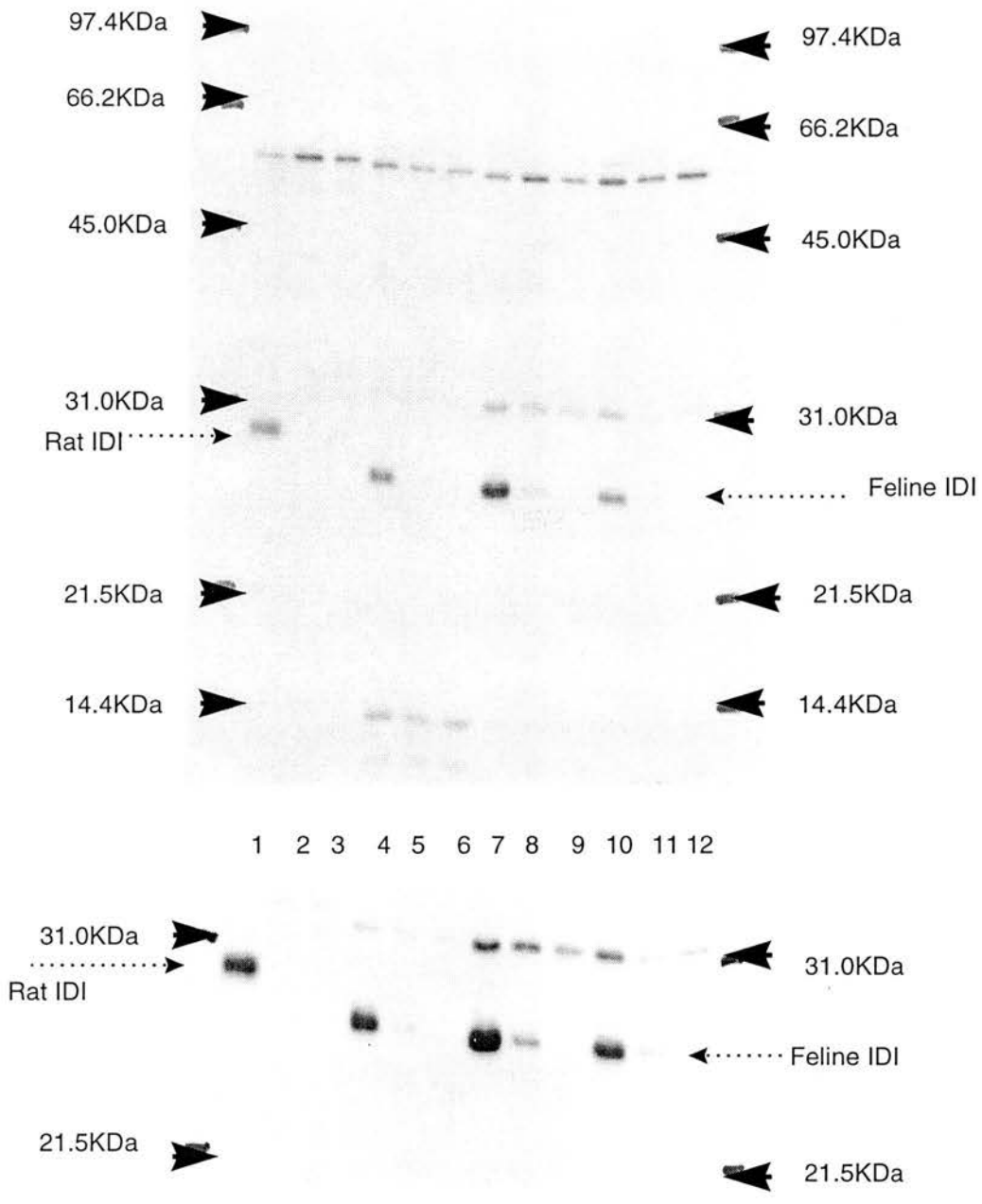


Figure 3.06: Lineweaver-Burk Plots showing T4 deiodination by cat liver homogenates. Experiments using 3 different liver homogenates are shown.



Close up view of 14 to 30 KDa region of the above autoradiograph of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat and feline liver microsomes.

Figure 3.07: Autoradiograph of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat and feline liver microsomes. Lanes: 1, rat liver microsomes; 2, rat liver microsomes plus PTU; 3, rat liver microsomes plus PTU plus rT3; 4, 7, 10, cat liver microsomes; 5, 8, 11, cat liver microsomes plus PTU; 6, 8, 12, cat liver microsomes plus PTU plus rT3. PTU, 1mM propylthiouracil; rT3, 6nM reverse T3.

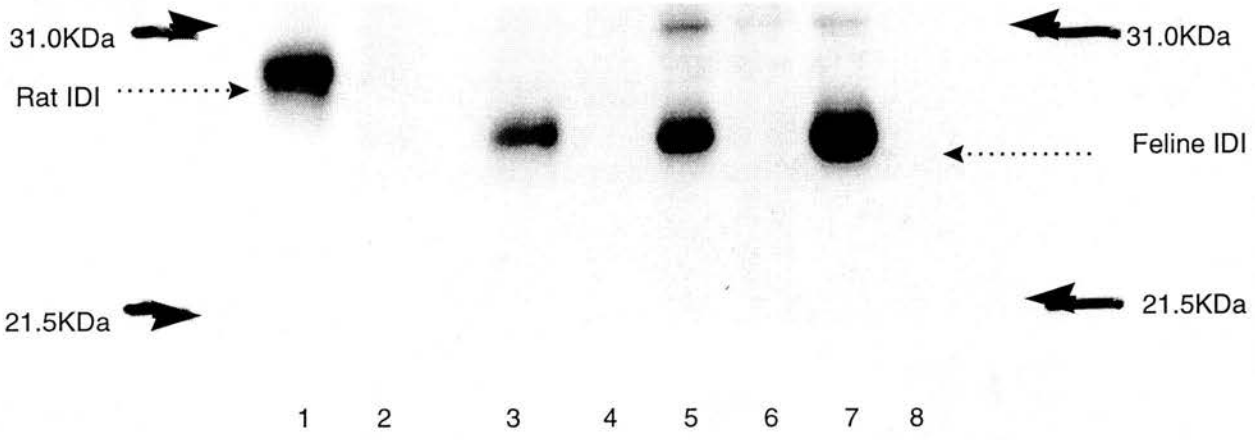
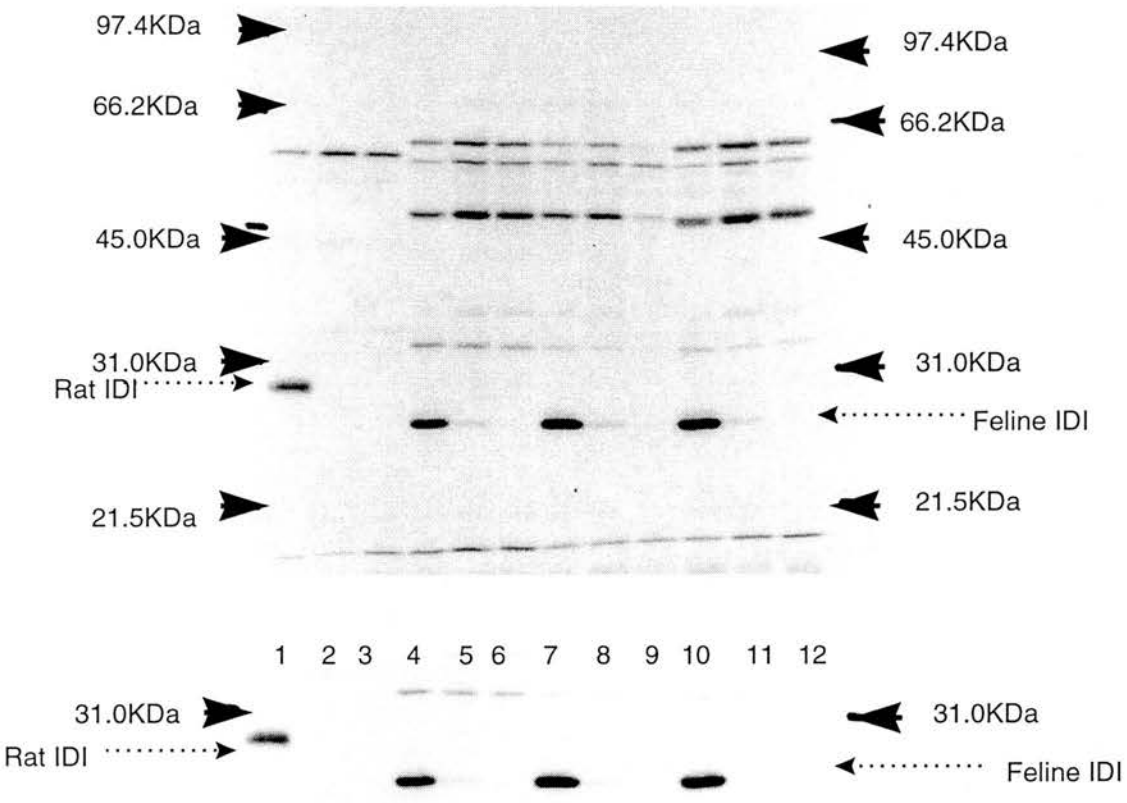


Figure 3.08: Autoradiograph of of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat and feline liver microsomes. Lanes: 1, rat liver microsomes; 2, rat liver microsomes plus AuG; 3, 5, 7, cat liver microsomes; 4, 6, 8, cat liver microsomes plus AuG. AuG, 12mM aurothioglucose.



Close up view of 14 to 30 KDa region of the above autoradiograph of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat liver and feline kidney microsomes.

Figure 3.09: Autoradiograph of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat and feline kidney microsomes. Lanes: 1, rat liver microsomes; 2, rat liver microsomes plus PTU; 3, rat liver microsomes plus PTU plus rT3; 4, 7, 10, cat kidney microsomes; 5, 8, 11, cat kidney microsomes plus PTU; 6, 8, 12, cat kidney microsomes plus PTU plus rT3. PTU, 1mM propylthiouracil; rT3, 6nM reverse T3.

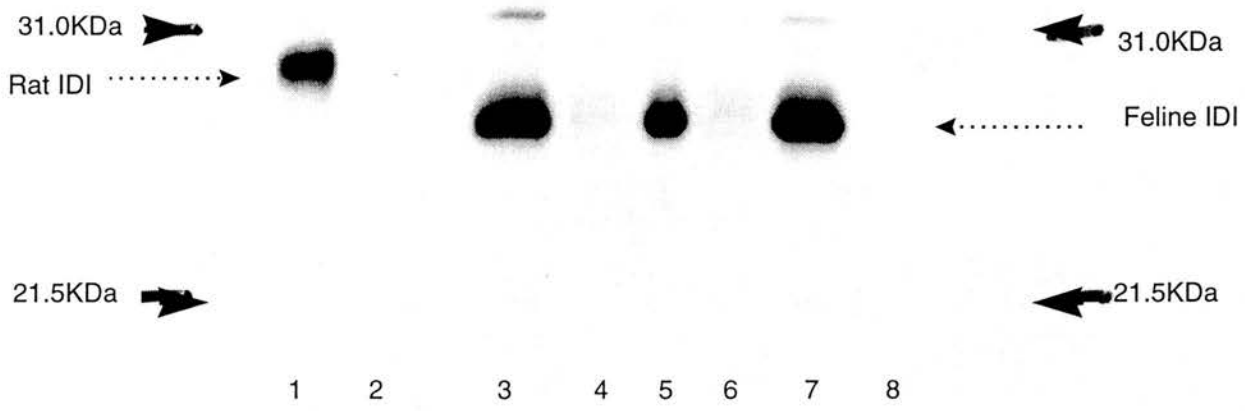
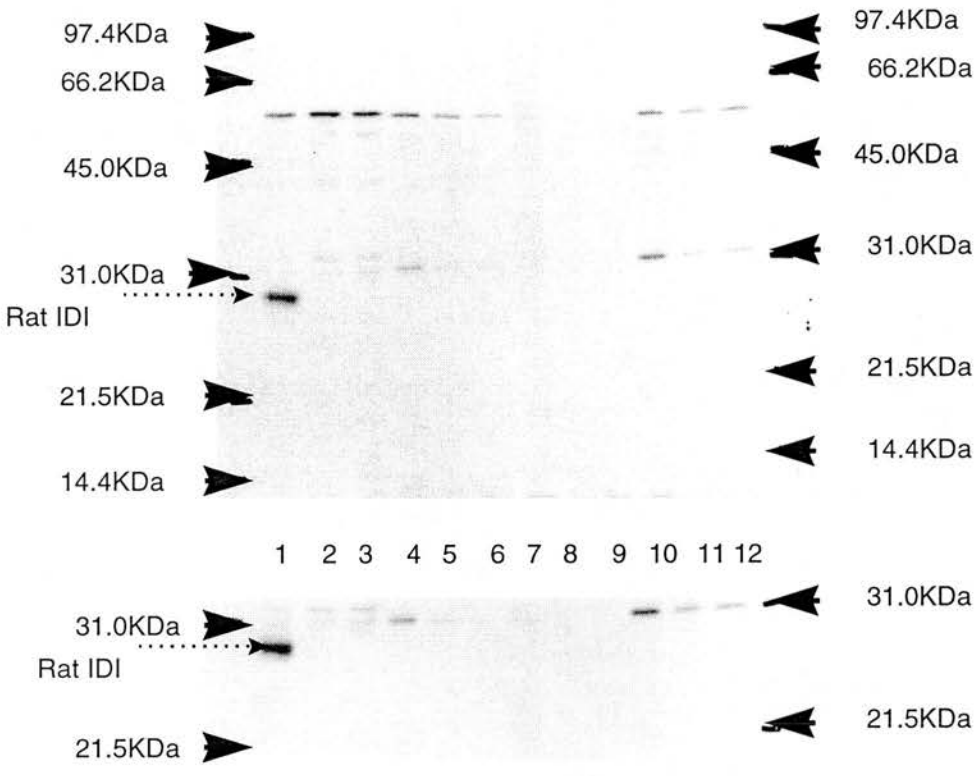


Figure 3.10: Autoradiograph of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat and feline kidney microsomes. Lanes: 1, rat liver microsomes; 2, rat liver microsomes plus AuG; 3, 5, 7, cat kidney microsomes; 4, 6, 8, cat kidney microsomes plus AuG. AuG, 12mM aurothioglucose.



Close up view of 14 to 30 KDa region of the above autoradiograph of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat liver and feline thyroid microsomes.

Figure 3.11: Autoradiograph of of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat and feline thyroid microsomes. Lanes: 1, rat liver microsomes; 2, rat liver microsomes plus PTU; 3, rat liver microsomes plus PTU plus rT3; 4, 7, 10, cat thyroid microsomes; 5, 8, 11, cat thyroid microsomes plus PTU; 6, 8, 12, cat thyroid microsomes plus PTU plus rT3. PTU, 1mM propylthiouracil; rT3, 6nM reverse T3.

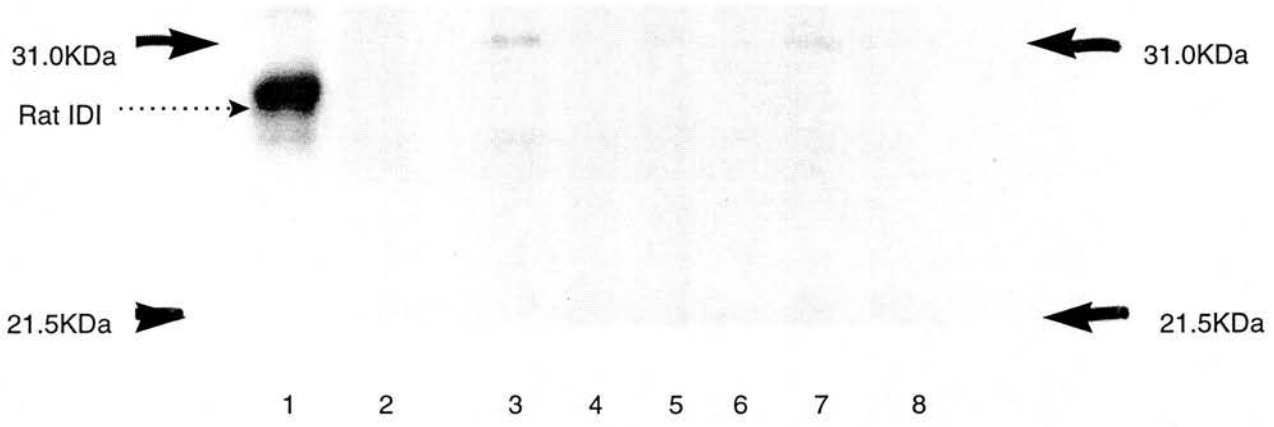
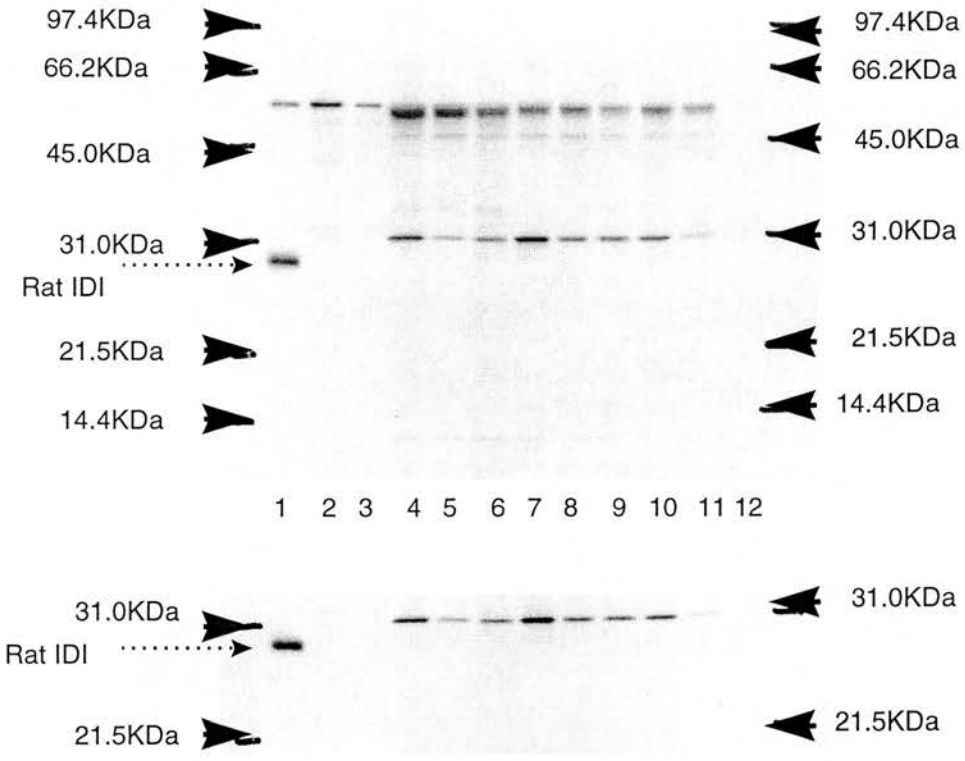


Figure 3.12: Autoradiograph of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat and feline thyroid microsomes. Lanes: 1, rat liver microsomes; 2, rat liver microsomes plus AuG; 3, 5, 7, cat thyroid microsomes; 4, 6, 8, cat thyroid microsomes plus AuG. AuG, 12mM aurothioglucose.



Close up view of 14 to 30 KDa region of the above autoradiograph of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat liver and feline brain microsomes.

Figure 3.13: Autoradiograph of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat and feline brain microsomes. Lanes: 1, rat liver microsomes; 2, rat liver microsomes plus PTU; 3, rat liver microsomes plus PTU plus rT3; 4, 7, 10, cat brain microsomes; 5, 8, 11, cat brain microsomes plus PTU; 6, 8, 12, cat brain microsomes plus PTU plus rT3. PTU, 1mM propylthiouracil; rT3, 6nM reverse T3.

Figure 3.14: Autoradiograph of SDS-PAGE showing ^{125}I-BArT3 affinity labelled proteins in rat and feline brain microsomes. Lanes: 1, rat liver microsomes; 2, rat liver microsomes plus AuG; 3, 5, 7, cat brain microsomes; 4, 6, 8, cat brain microsomes plus AuG. AuG, 12mM aurothioglucose.

3.04 DISCUSSION

The iodothyronine deiodinases play a crucial role in regulating thyroid hormone metabolism in many tissues. The expression of the selenoenzyme IDI has been studied extensively in humans and rats and such work has suggested that this enzyme provides the major source of plasma T3. However, the observation that the preferred substrate for IDI in these species is rT3 and not T4 has led some to suggest that one of the major roles of IDI may be to metabolise rT3 (Visser, Kaptein, Terpstra *et al.,* 1988). More limited studies of IDI have been reported in other species but the use of affinity labelling with ^{125}I- BrAc-rT3 has suggested that the hepatic expression of IDI is highest in the rat and dog, with humans, mice, rabbits, cows, pigs, sheep, goats, chickens and ducks having a similar level of expression (Schoenmakers, Pigmans and Visser, 1992). Activity measurements of IDI with rT3 as substrate have shown that there are also clear species differences in the turnover number of hepatic IDI. Rats, rabbits and goats have the highest turnover number whilst dogs have the lowest, being approximately 10 times lower than that found in the rat. No studies on feline IDI have been reported.

The results reported here show that although certain characteristics of hepatic and renal IDI are similar in the cat and rat, the substrate specificity of the enzyme between these species is clearly different. The kinetics and ^{125}I-BrAc-rT3 affinity labelling of this enzyme have also been examined in the present study.

Affinity labelling of hepatic microsomes using ^{125}I-BrAc-rT3 demonstrated that the feline enzyme was expressed at similar levels to that found in the rat, although the feline enzyme had an Mr which was approximately 3 KDa smaller than the rat enzyme. Such modest differences in the Mr of IDI have been reported previously between many species (Schoenmakers, Pigmans and Visser, 1992). The sensitivity of the rat and feline enzyme to PTU and AuG was also similar in that both these agents prevented affinity labelling of IDI by ^{125}I-BrAc-rT3. The feline kidney also expressed levels of IDI similar to that found in feline liver as assessed by affinity labelling.

When the activity of IDI was compared in rat and feline liver homogenates using T4 as substrate, we found that the relative activities of the enzyme were not significantly different between the species (Table 3.01). More detailed kinetic analysis showed that the Km for T4 of the rat enzyme was approximately only three times lower than the feline enzyme. However, the Vmax of the enzyme in cat and rat liver was not significantly different, which was in agreement with the affinity

labelling data that also suggested that similar masses of enzyme were present in each of the tissues.

The use of rT3 as substrate showed marked differences in the ability of rat and feline IDI to metabolise this substrate. The rate at which feline liver homogenates metabolised rT3 under the standard assay conditions employed (6nM) was approximately 0.2 per cent of the reaction rate found in rat homogenate. This low activity was inhibited by AuG and PTU. With rT3 as substrate it was difficult to determine a precise and accurate estimate of Km in feline liver. This was because the Km for this substrate appeared to be similar to the highest concentration of substrate that could be obtained in solution. However, at a conservative estimate, the Km of the feline enzyme was 7.5 fold higher than the enzyme in rat liver. This finding is similar to reports on the characteristics of canine IDI. Laurberg and Boye (1982) have reported that dog IDI metabolises rT3 much less effectively than rat IDI, and rT3 cannot inhibit *in vitro* deiodination of T4 unless the concentration of rT3 is greater than that of T4. Additionally, Schoenmakers, Pigmans and Visser (1992) demonstrated that rT3 was a less effective inhibitor of ^{125}I-BrAc-3,5,3'-triiodothyronine labelling in dogs than in humans and rats.

After successful cloning of human, canine and rat IDI cDNAs (Toyoda, Harney, Berry *et al.,* 1994), it was established that there was significant sequence homology between the IDI cDNA of humans (81%) and rats (76%) to the cDNA of dog IDI. The TGA selenocysteine codon (considered to be the active site of IDI) was identically positioned in all three species (400 to 402), and the area surrounding the active site was well conserved (Toyoda, Harney, Berry *et al.,* 1994). The two histidine residues (153 and 169), previously regarded as essential for enzyme function, were also conserved in dog IDI (Toyoda, Harney, Berry *et al.,* 1994). The most obvious differences were the absence of a highly-conserved (human and rat) 5 amino acid segment in the amino-terminal portion in dog IDI and that canine IDI was 10-fold less sensitive to PTU, and 4-fold less sensitive to AuG inhibition, than human IDI (Toyoda, Harney, Berry *et al.*, 1994). This group concluded that the substitution of histidine for phenylalanine in the dog enzyme resulted in important changes to the binding affinity for rT3. Whilst this may be true in dogs, the different kinetics between feline and rat IDI do not appear to be the result of poor affinity for rT3 as our results show that ^{125}I-BrAc-rT3 labelling is similar between cats and rats.

These data suggest that in the cat, a major source of circulating T3 is likely to arise from hepatic deiodination of T4. Indeed, the concentrations of serum T3 in the rat and the cat are similar (Beckett, Beddows, Maurice *et al.,* 1987; Rutgers, Heusdens and Visser, 1991). The data further

suggest that hepatic metabolism of rT3 by IDI cannot proceed at a significant rate in the cat. However, as the serum concentrations of rT3 were significantly lower in the cat (median, 0.164nmol/l; range, 0.086 to 0.244nmol/l) than the rat (median, 0.3305nmol/l; range 0.2670 to 0.3710nmol/l), this suggests that either the major fraction of serum rT3 does not arise from the liver in the cat, or that metabolism of rT3 must occur by alternative pathway(s). A clear candidate for such a pathway is prior sulphation with subsequent deiodination of sulphated rT3 (Rutgers, Heusdens and Visser, 1991).

In rats and humans, sulphation of thyroid hormones is considered to be an important regulator of thyroid hormone metabolism. In these species, sulphation of some iodothyronines (T4 and T3, but not rT3) accelerates deiodination by IDI compared to non-sulphated iodothyronines (Visser, Mol and Otten, 1983; Rutgers, Heusdens and Visser, 1991). This has led to the suggestion that a balance between the expression of the sulphotransferases, sulphatases and deiodinases involved in thyroid hormone metabolism may be important regulators of thyroid hormone expression during foetal development (Richard, Hume, Kaptein *et al.,* 1998). In the present study, the ability of feline IDI to metabolise rT3 sulphate or the ability of feline liver to synthesise rT3 sulphate was not investigated but clearly this would be of interest. Whilst sulphation prior to deiodination does not appear to alter the deiodination rate of rT3 in rats and humans, it is unknown whether sulphation may accelerate rT3 deiodination in the cat.

Using affinity labelling and activity assays, this study has shown that IDI is not expressed in the thyroid of the cat. In this regard, cats are similar to goats, cattle, sheep, rabbits, pigs, llama and deer. In contrast, thyroidal IDI is strongly expressed in humans, dogs, rats, mice and guinea pigs (Beckett, Beech, Nicol *et al.,* 1993) and, in these species, expression of the enzyme appears to be regulated through activation of the cyclic AMP cascade through changes in adenylate cyclase activity. When it is expressed, thyroidal IDI appears to provide a significant source of circulating T3 particularly when activation of the TSH receptor is initiated such as in hypothyroidism via TSH or in Graves' disease through thyroid stimulating immunoglobulins. Clearly therefore, in the cat, thyroidal deiodination cannot be involved in an adaptive response to hypothyroidism. Spontaneous adult-onset hypothyroidism in the cat is known to be extremely rare (Rand, Levine, Best *et al.,* 1993).

In conclusion, these data suggest that in the cat, plasma T3 originates largely from the deiodination of T4 in the liver and kidney but clearance of rT3 must proceed by a metabolic route that is not dependent on deiodination of the unsulphated molecule.

4.00 CONTROL OF GROWTH OF THE NORMAL AND ADENOMATOUS FELINE THYROCYTE

4.01 INTRODUCTION

The control of growth and function of thyrocytes involves the interaction of numerous hormones with receptors including the TSH receptor. Receptor activation and subsequent stimulation of growth and function is species-specific and will also vary within individual thyroid glands due to the natural heterogeneity of thyrocytes. Despite thyrotoxicosis being the most common endocrine disorder of cats and the similarities between feline and human toxic nodular goitre, little work has been carried out regarding the control of growth in normal feline thyrocytes.

Peter *et al.* (1987) first reported on the autonomous nature of feline thyrocytes from cats with hyperthyroidism in an *in vivo* model, transplanting thyroid nodules from hyperthyroid cats into nude (nu/nu ICR) mice. They demonstrated that both growth, assessed by [^{3}H]-TDr uptake, and function, assessed by ^{131}iodine uptake, were independent of TSH. No stimulation of growth or function was noticed following injection of mice with sera from hyperthyroid cats.

Only two reports on the characteristics of growth and function of autonomous feline thyrocytes *in vitro* has been published (Gerber, Peter, Bosiger *et al.,* 1991; Peter, Gerber, Studer *et al.,* 1991). Using isolated follicles (from cats affected with thyrotoxicosis) grown in a collagen matrix, Peter *et al.* (1991) demonstrated that both growth (assessed by [^{3}H]-TDr incorporation) and function (assessed by ^{131}iodine uptake) of adenomatous cells were independent of TSH. Furthermore, these characteristics were spontaneously increased in adenomatous tissue compared to normal tissue that surrounded these nodules, suggesting they were truly autonomous. In their report, EGF was the most potent growth factor followed by calf serum, whilst TSH, IGF-1 and insulin had no effect. Gerber *et al.* (1991) studied the growth characteristics of thyrocytes in monolayer isolated from cats with hyperthyroidism. They concluded that there were widely different growth characteristics to TSH, EGF, retinoic acid and iodine between preparations. None of the feline cells responded to TSH, but four out of five demonstrated growth stimulation with EGF and retinoic acid. Three out of five grew in response to iodine.

Brown *et al.* (1992), demonstrated that purified serum IgG from hyperthyroid cats stimulated growth of FRTL-5 cells and displaced binding of TSH from its receptor using porcine and feline thyrocyte membranes. It therefore appeared that there was a humoral factor which bound to the TSH receptor that was possibly involved in the pathogenesis of feline hyperthyroidism. Brown's group, however, did not use the optimal model system, i.e. feline thyrocytes, to test this conclusion in the species of interest.

In vitro models can be used to infer characteristics of growth and function of thyrocytes *in vivo* and indeed such models have been widely employed to study growth of thyrocytes in a wide range of species. The main disadvantage with using primary cells for determination of factors involved with growth is the variability between preparations. Thyroid glands removed from cats undergoing thyroidectomy are diseased and the ratio of diseased (adenomatous) to 'normal' tissue will vary between each gland. It may, therefore, be preferable to use normal glands. Transformed cell lines such as the FRTL-5 and cells expressing recombinant functional TSH receptors (JPO9/2), whilst being more consistent in responses to growth factors, do not behave as normal thyroid cells. *In vitro* experiments suffer further because they do not mimic the *in* vitro system where autocrine and paracrine factors can interact. Despite these disadvantages, cell culture systems are widely used to study thyrocyte growth.

The aims of this study were:

- to test [^{3}H]-TDr incorporation into DNA as a suitable method to detect growth in feline thyrocytes and to investigate the growth response to known thyrotropic growth factors (bTSH, EGF, IGF-1) in order to investigate some of the growth responses of feline thyrocytes and.
- to examine the growth response of FRTL-5 cells and feline thyrocytes to purified IgG from euthyroid and thyrotoxic cats to test the hypothesis that hyperthyroid cat serum contains humoral factors which stimulate growth of thyrocytes.

4.02 METHODS

a) ISOLATION AND MAINTENANCE OF FELINE THYROCYTES AND EXPERIMENTAL DESIGN FOR GROWTH EXPERIMENTS

Feline thyrocytes were harvested as described in Chapter 2 from euthyroid cats euthanased for reasons other than hyperthyroidism (FT), and from thyrotoxic cats undergoing surgical thyroidectomy for the treatment of the disease (FNG). Cells were maintained in RPMI 1640, supplemented with L-glutamine (2mmol) and penicillin/streptomycin (100U/ml and 100µg/ml respectively), and passaged as required (usually weekly) with 1x trypsin-EDTA. Cells were plated into 12 well plates for growth assays at a density of $1x10^4$ cells/ml. For all experiments, cells were below passage number two.

For experiments involving purified IgG, only normal feline thyrocytes were used. Feline IgG (1mg/ml final concentration) dialysed against RPMI 1640 medium for 4 days was added to cells in triplicate 12 well plates. NCS (5% final concentration), L-glutamine and penicillin/streptomycin were added as above. The medium from each well was removed at day 3, and an aliquot of the medium with purified IgG was replaced along with L-glutamine and penicillin/streptomycin. The total cell incubation time for growth experiments was 7 days. This is similar to the method used by Brown *et al.* (1990). At the end of day 6, 0.5µCi[^{3}H]-TDr/ml of medium was added to each well (0.5µCi[^{3}H]-TDr/well) and the cells were left to incubate overnight to allow incorporation of the tritiated thymidine.

b) MAINTENANCE OF FRTL-5 CELLS AND EXPERIMENTAL DESIGN FOR GROWTH EXPERIMENTS

FRTL-5 cells were maintained as described in Chapter 2.038. Cells were deprived of bTSH for 5 days before experiments were undertaken and were below passage 10 for all experiments. Purified IgG (1mg/ml final concentration) dialysed against Coon's F-12 medium for 48 hours was added to cells in triplicate 12 well plates. "6H" mix and NCS (5% final concentration) were added to each well to achieve a concentration of "6H" mix that was used for maintenance of this cell line. Medium was replaced (along with purified IgG) after 3 days. The total cell incubation time for

growth experiments was 7 days. At the end of day 6, 0.5μCi[^{3}H]-TDr/ml of medium was added to each well (0.5μCi[^{3}H]-TDr/well) and the cells were left to incubate overnight. Growth was assessed by incorporation of [^{3}H]-TDr over 12 hours into DNA as described in Chapter 2.

c) STATISTICAL ANALYSIS

ANOVA was used to test for significant differences in growth response between doses of bTSH, EGF in comparison to basal. The Mann-Whitney U test was used to test for significant differences between groups (i.e. euthyroid and hyperthyroid cats).

4.03 RESULTS

a) GROWTH RESPONSE OF NORMAL AND ADENOMATOUS FELINE THYROCYTES TO bTSH, EGF AND IGF-1.

i) Growth response to bTSH

At doses from 0.001μU/ml to 100mU/ml, bTSH failed to produce a significant growth response in one normal thyroid preparation (FT5) (Fig. 4.01) and two adenomatous preparations [FNG 26 (Fig. 4.02) & 29]. At 1.0μU/ml bTSH, a statistically reduced growth response from basal occurred in one FT preparation (FT5, Figure 4.01). At doses from 0.001μU/ml to 500mU/ml bTSH, failed to produce a significant growth response in all of eight adenomatous preparations (FNG 16 to 20 and 23 to 25) but a small but significant response was seen at a dose of 1000mU/ml. Growth responses at this very high concentration of bTSH may be due to growth factor contaminants in the pituitary-derived preparation of bTSH. Accumulated growth data using bTSH in each of the eight adenoma preparations are represented in Figure 4.03 and show that overall, only a bTSH concentration of 1000mU/ml gave a significant stimulation of approximately two fold.

ii) *Growth response to EGF and IGF-1*

EGF at doses from 1ng/ml to 200ng/ml produced a small but significant growth of a normal feline thyrocyte preparation at 10 and 100ng/ml. IGF-1 at doses of 0.1 - 5ng/ml also produced a significant growth response in one normal thyrocyte preparation (FT5) (Figure 4.04). In an adenomatous thyrocyte preparation (FNG 26), EGF produced an approximately four fold increase in growth at doses from 10 to 200ng/ml. IGF-1 at doses from 0.1 to 5ng/ml also significantly stimulated growth in feline adenomatous thyrocytes (FNG 23, 24, 25, 26, 29), but to a lesser extent than the normal thyrocyte preparation (Figure 4.05).

b) GROWTH RESPONSE OF FRTL-5 TO bTSH AND PURIFIED IgG (1mG/ml) FROM EUTHYROID AND HYPERTHYROID CATS.

i) Growth response to bTSH (0.001mU/ml to 100mU/ml).

Before experiments testing growth response of FRTL-5 cells in response to purified IgG were undertaken, the growth response to bTSH was tested. This was necessary as it is well known that FRTL-5 cells lose their TSH responsiveness over time and some strains respond to lower doses of TSH than others. There was a significant and linear dose response to bTSH from 0.001mU/ml - 100mU/ml ($p < 0.05$) (Fig. 4.06).

ii) Growth response to purified feline IgG (1mg/ml).

The growth response of FRTL-5 cells to purified IgG (1mg/ml) from euthyroid and hyperthyroid cats was examined. There was a significant growth response to 100mU/l bTSH, and in seven out of 10 (70%) euthyroid cats and six out of 11 (55%) hyperthyroid cats ($p < 0.05$)(Fig. 4.07) when purified IgG was used. Euthyroid cat IgG samples as a group, produced a significantly higher growth response than hyperthyroid cat IgG samples ($p = 0.0258$)(Fig. 4.08) but the differences were small.

c) GROWTH RESPONSE OF NORMAL FELINE THYROCYTES TO PURIFIED IgG (1mG/ml) FROM EUTHYROID AND HYPERTHYROID CATS.

The growth response of normal feline thyrocytes to purified IgG (1mg/ml) from euthyroid and hyperthyroid cats was examined. bTSH was not used as a control in these cells as it had not been possible to demonstrate a growth response to it previously. There was a significant growth response to EGF (1ng/ml)($p = 0.000$) compared to basal. There was no significant growth response to purified IgG from euthyroid or hyperthyroid cats compared to basal ($p = 0.2620$ and 0.5508 respectively) (Fig. 4.09). However, the growth response in feline thyrocytes to hyperthyroid cat IgG was slightly but significantly higher than that observed with euthyroid cat IgG ($p = 0.0333$)(Fig. 4.10).

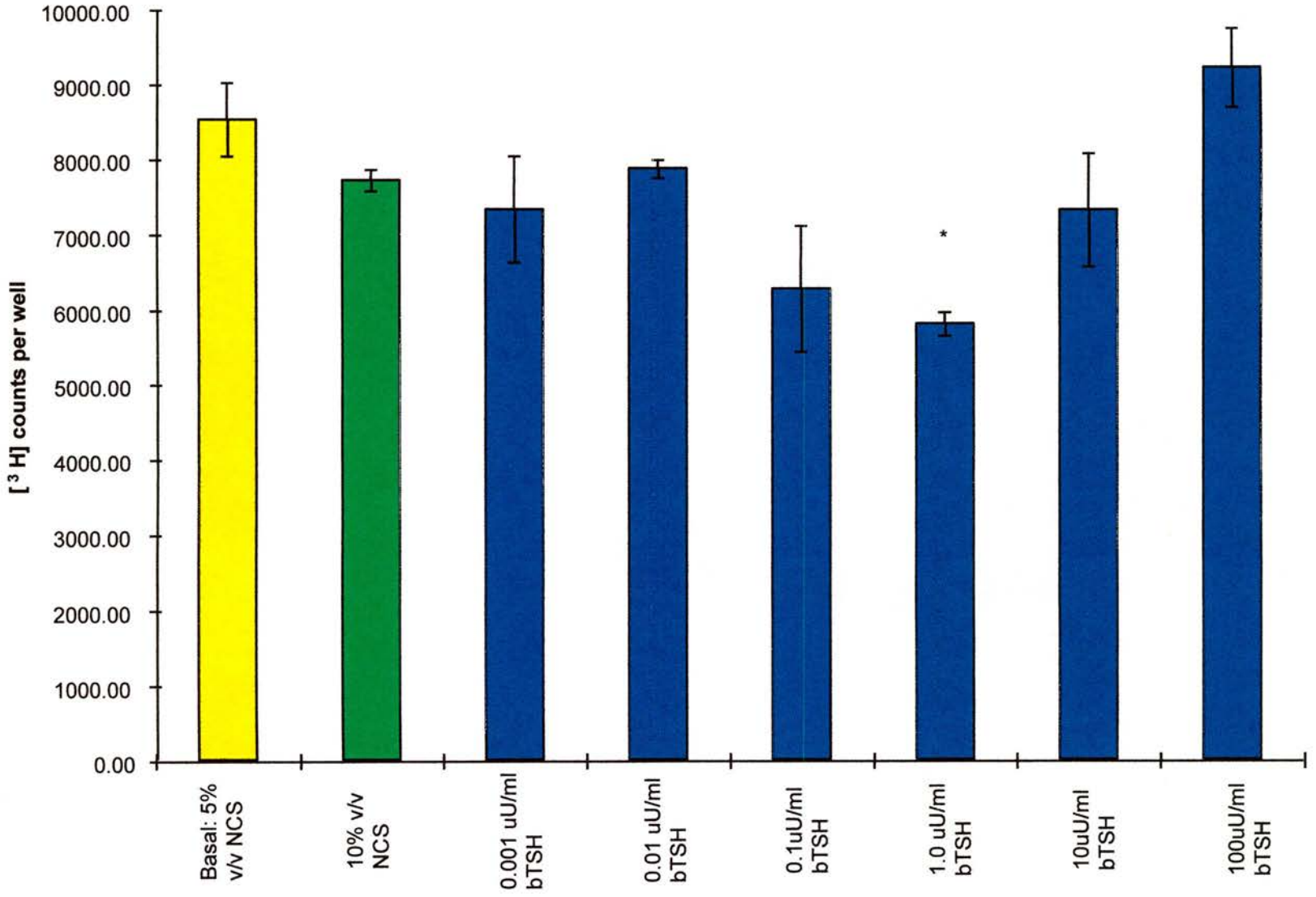


Figure 4.01: Growth of normal feline thyrocytes (FT5) in response to bovine thyrotropin stimulating hormone (bTSH). Results shown are the mean of triplicate wells ± SEM. $p<0.05$*. NCS, newborn calf serum.

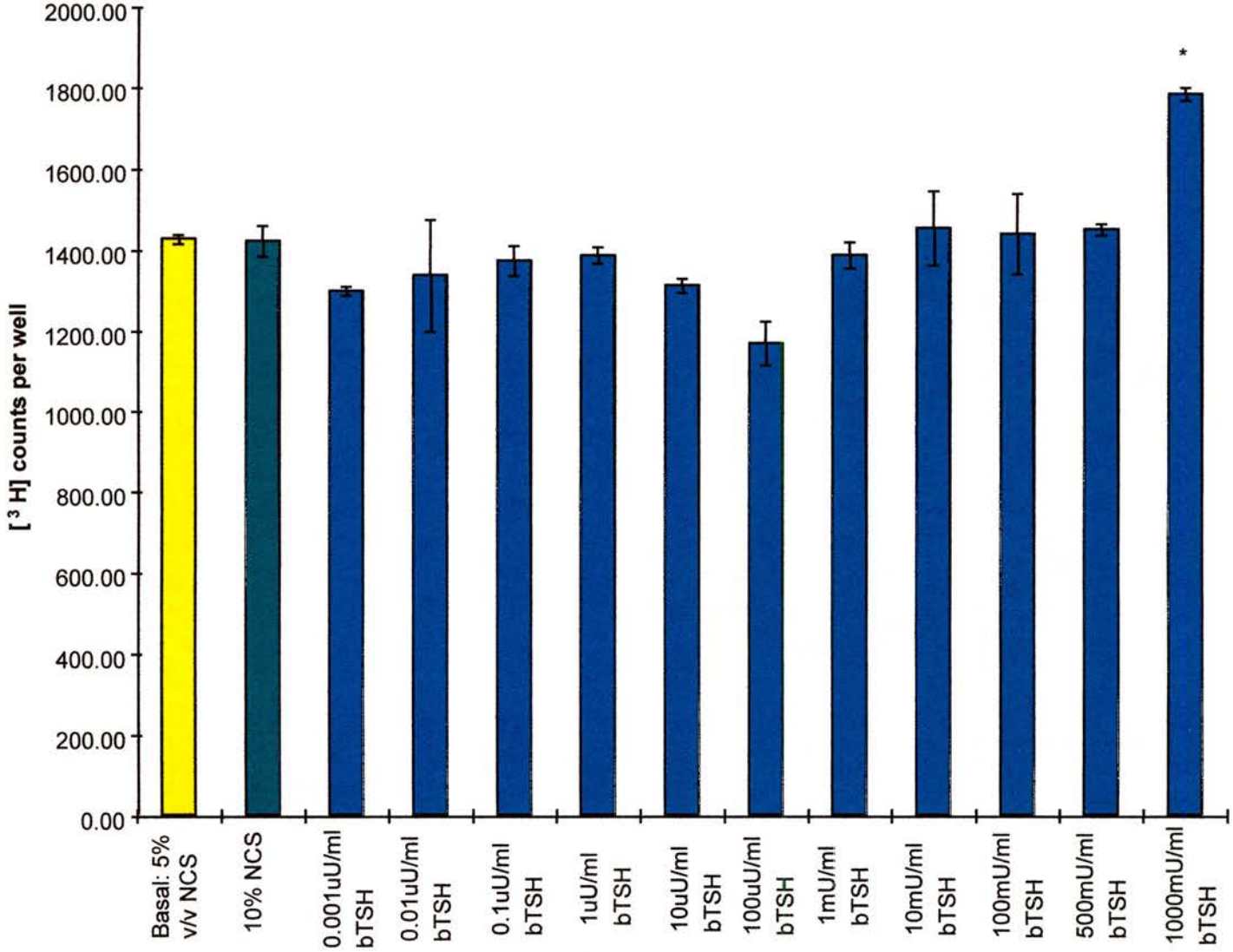


Figure 4.02 : Growth response of feline adenomatous cells (FNG26) to bTSH with 5% NCS. Results shown are those of mean of triplicate wells ± SEM. $p<0.05$*. NCS, newborn calf serum.

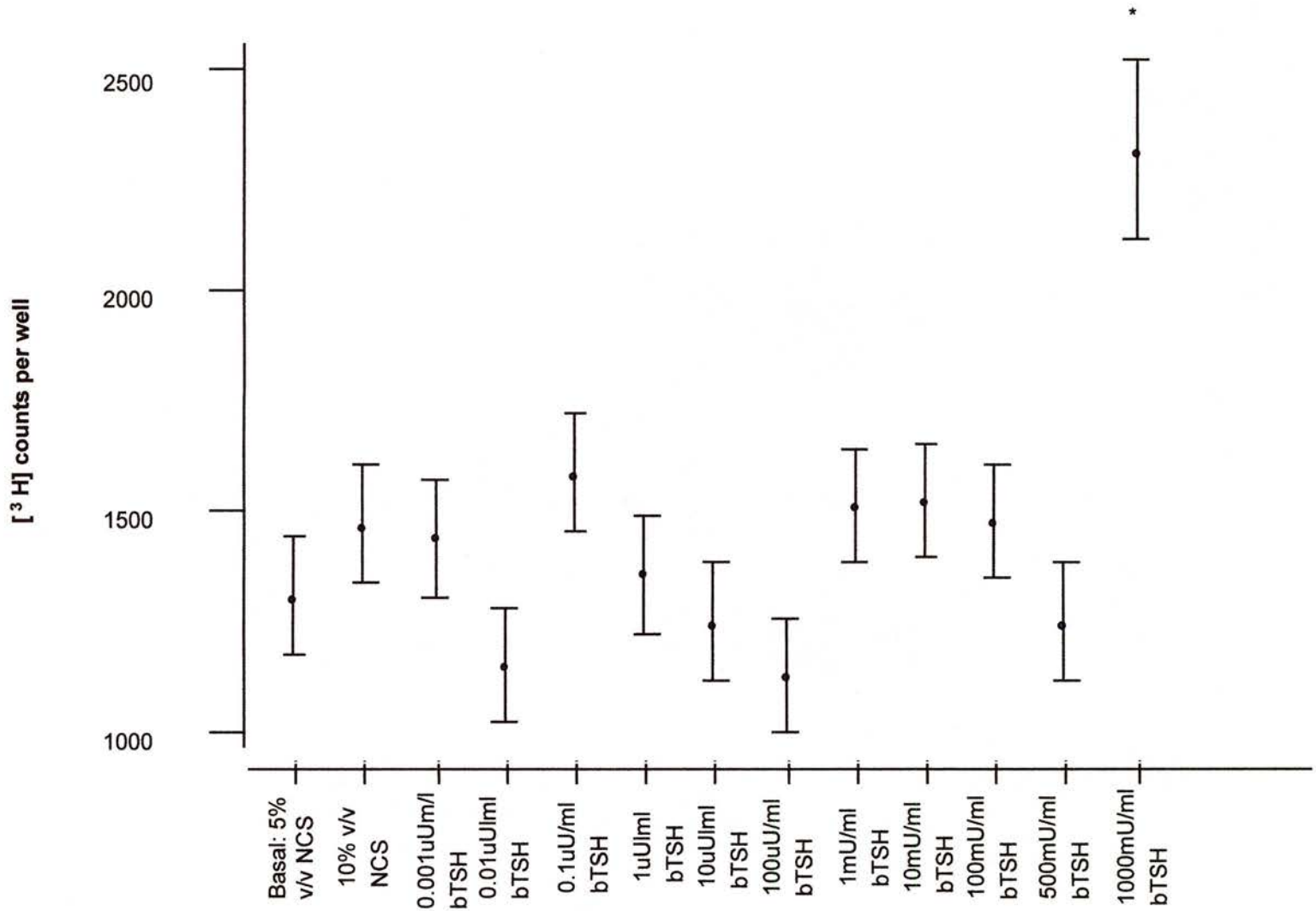


Figure 4.03 : Growth response of eight feline adenomatous cell preparations (FNG18-25) to bTSH with 5% NCS. Results shown are those of the mean of all eight preparations ± SEM. $p<0.05$*. NCS, newborn calf serum.

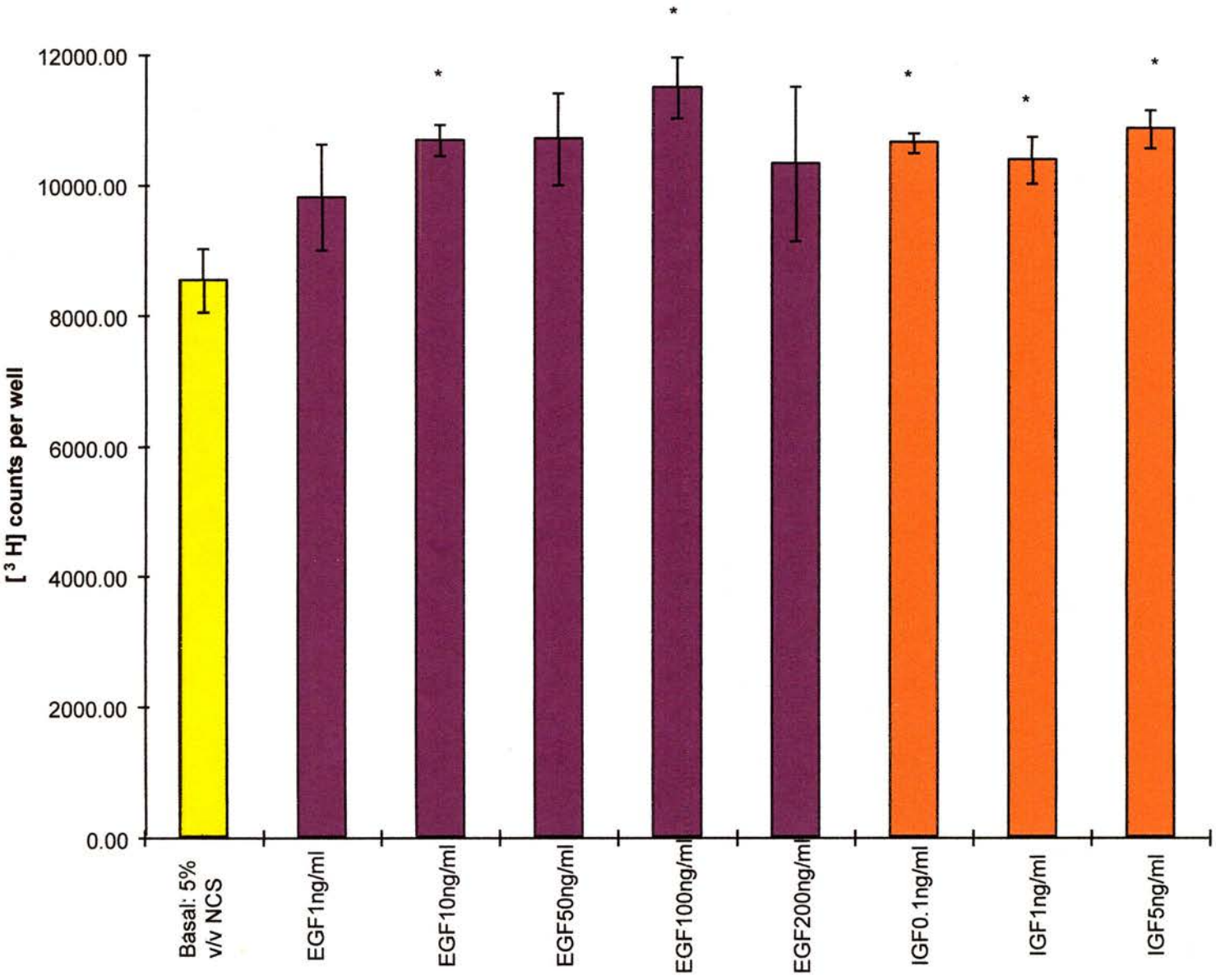


Figure 4.04: Growth of normal feline thyrocytes(FT5) in response to epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1). Results shown are those of triplicate wells ± SEM. $p<0.05$*. NCS, newborn calf serum.

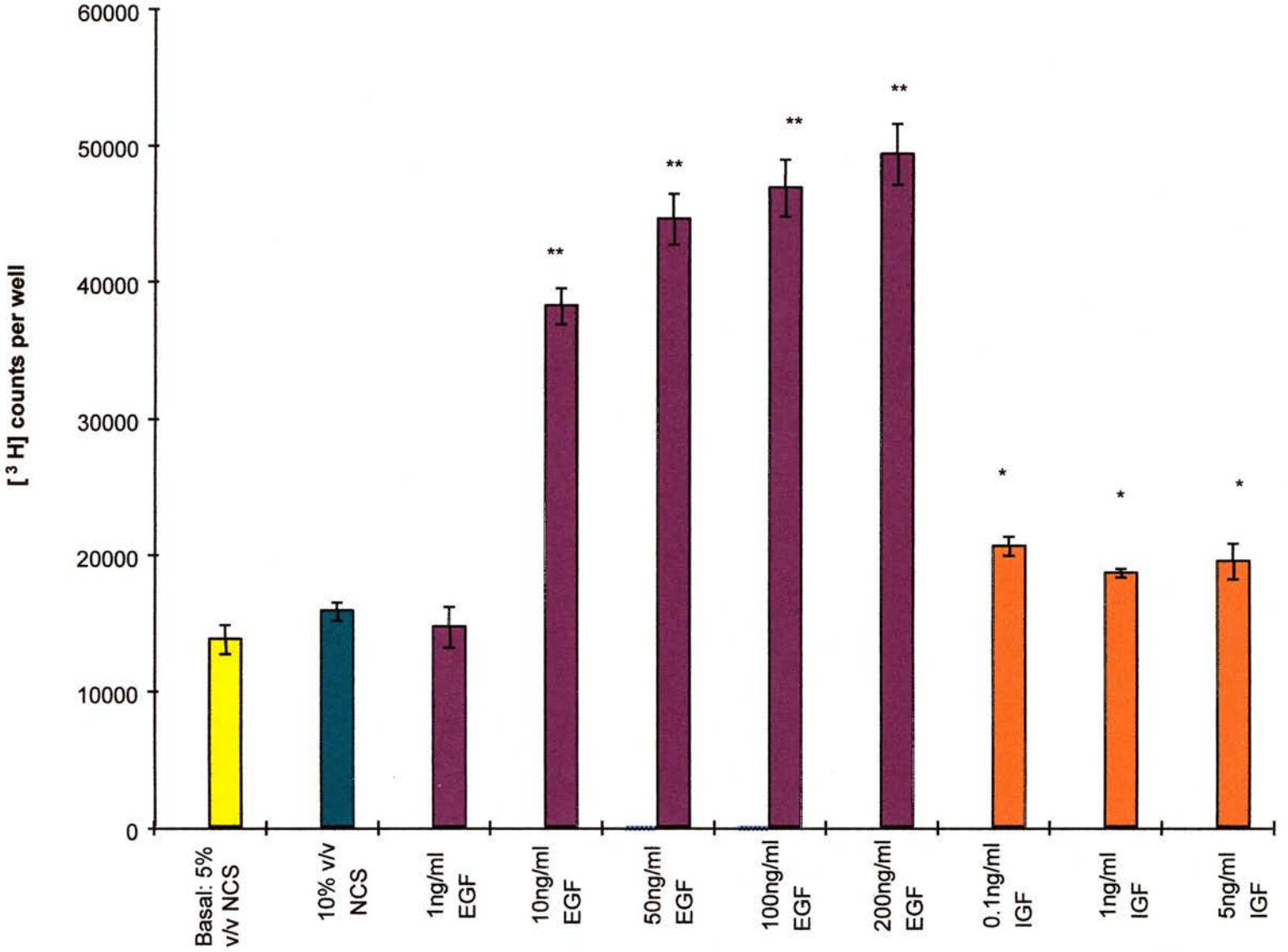


Figure 4.05: Growth response of feline adenomatous cells (FNG26) to epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) with 5% NCS. Results shown are those of mean of triplicate wells ± SEM. $p<0.05$*; $p<0.01$**. NCS, newborn calf serum.

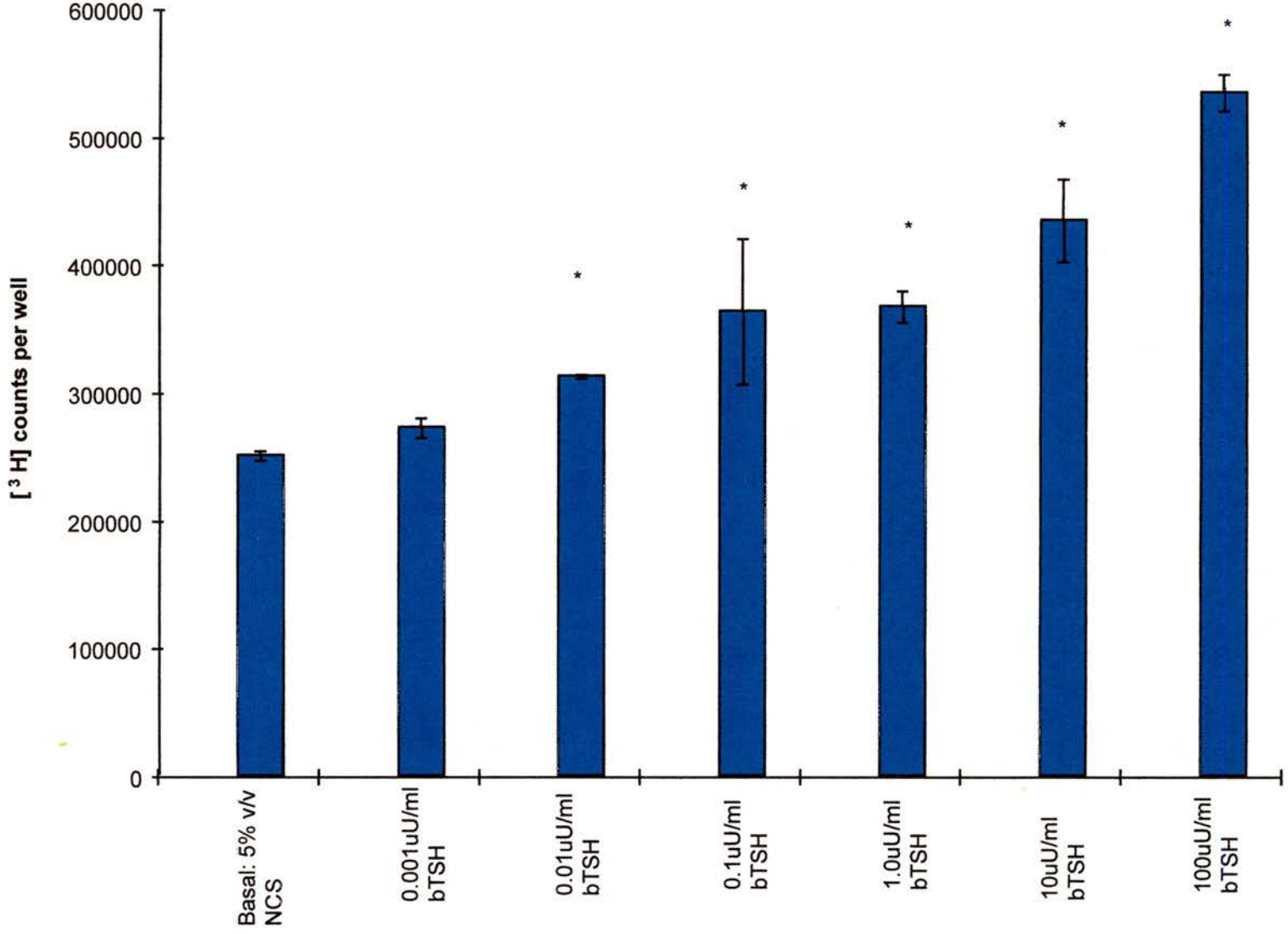


FIgure 4.06: Growth of FRTL-5 cells in response to bovine thyrotropin stimulating hormone (bTSH). Results shown are those of mean of all eight preparations ± SEM. $p<0.05$*. NCS, newborn calf serum.

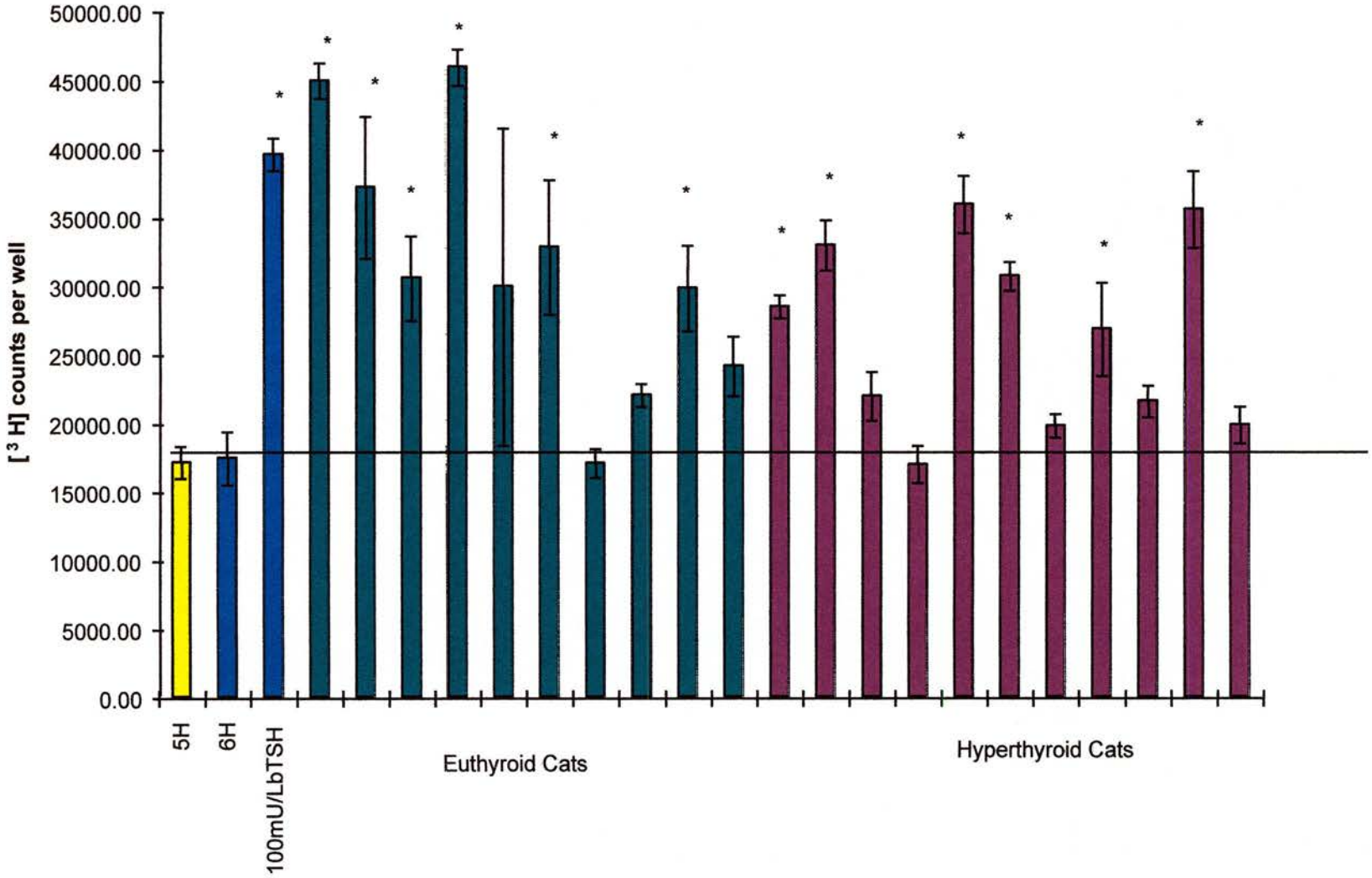


Figure 4.07 : Growth of FRTL-5 cells in response to purified IgG (1mg/ml). Results shown are the mean of triplicate wells ± SEM. $p<0.05$*. bTSH, bovine thyrotropin stimulating hormone; 5H, Coon's F-12 with '5H' mix; 6H, Coon's F-12 with '6H' mix.

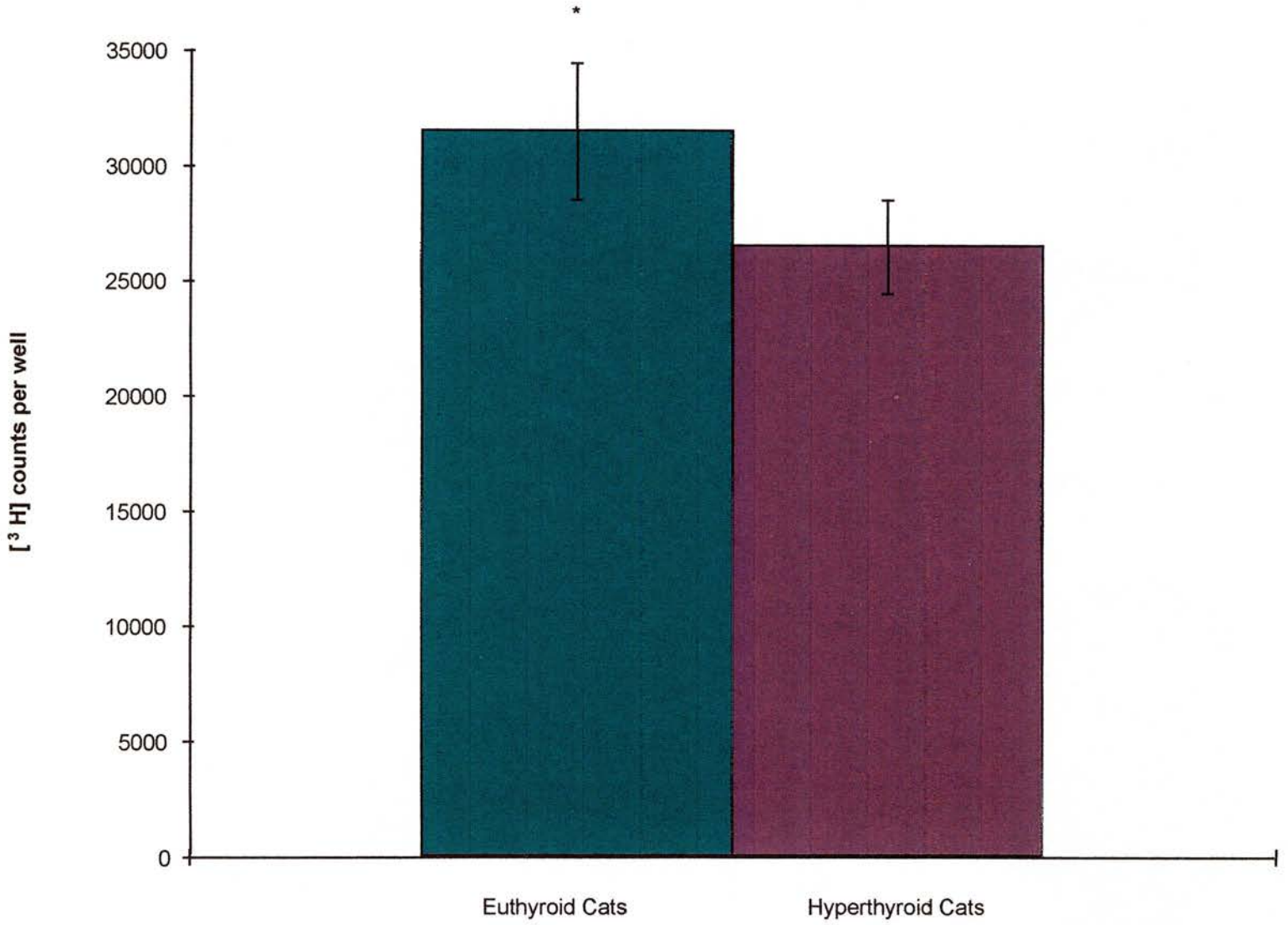


Figure 4.08 : Growth of FRTL-5 cells in response to purified IgG (1mg/ml) Comparison between euthyroid and hyperthyroid cats. Results shown are the mean of all cats in a group ± SEM. $p<0.05$*.

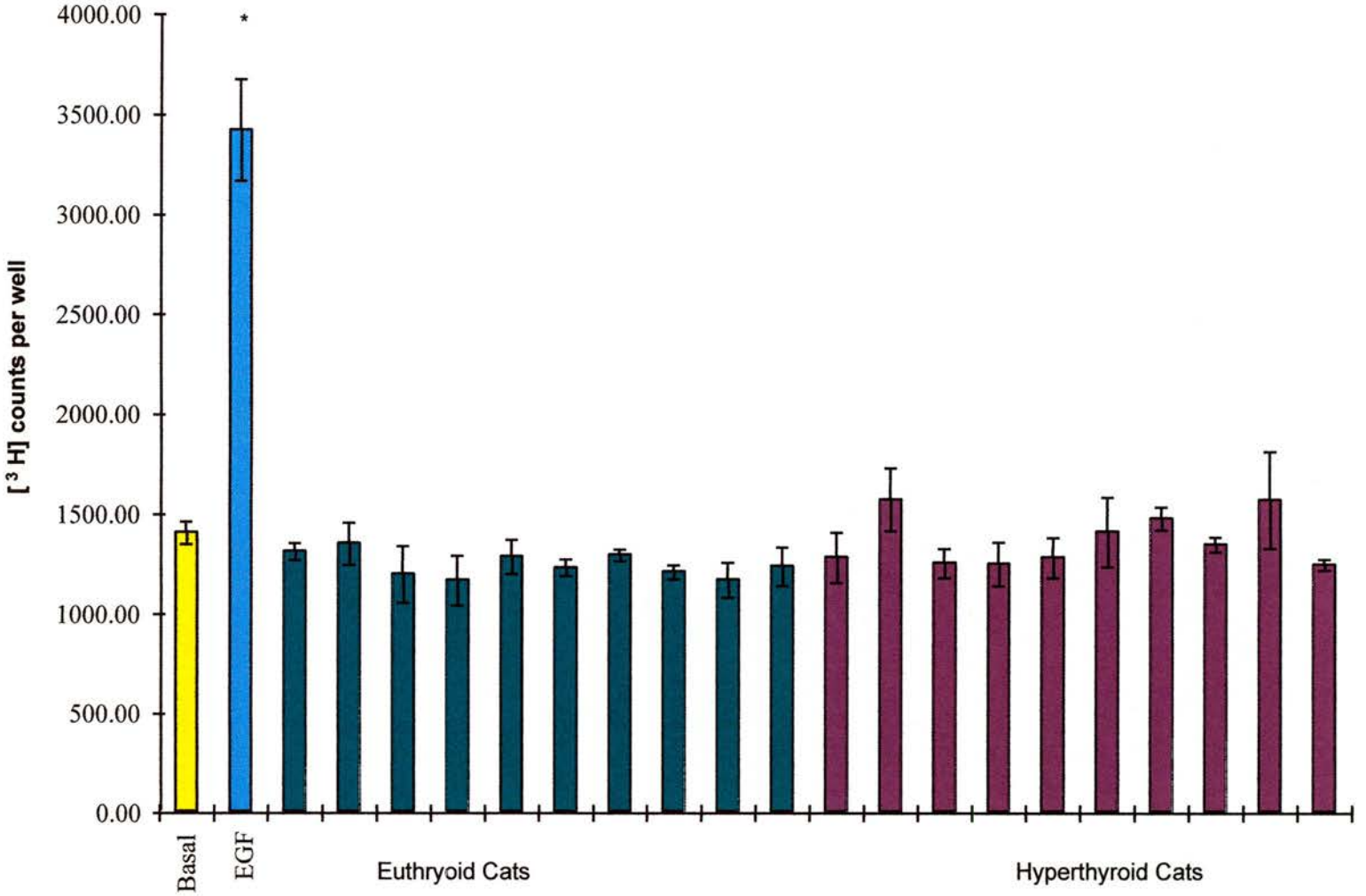


Figure 4.09: Growth of normal feline thyrocytes in response to purified IgG (1mg/ml) and epidermal growth factor (EGF). Results shown are the mean of triplicate wells ± SEM. $p<0.05$*.

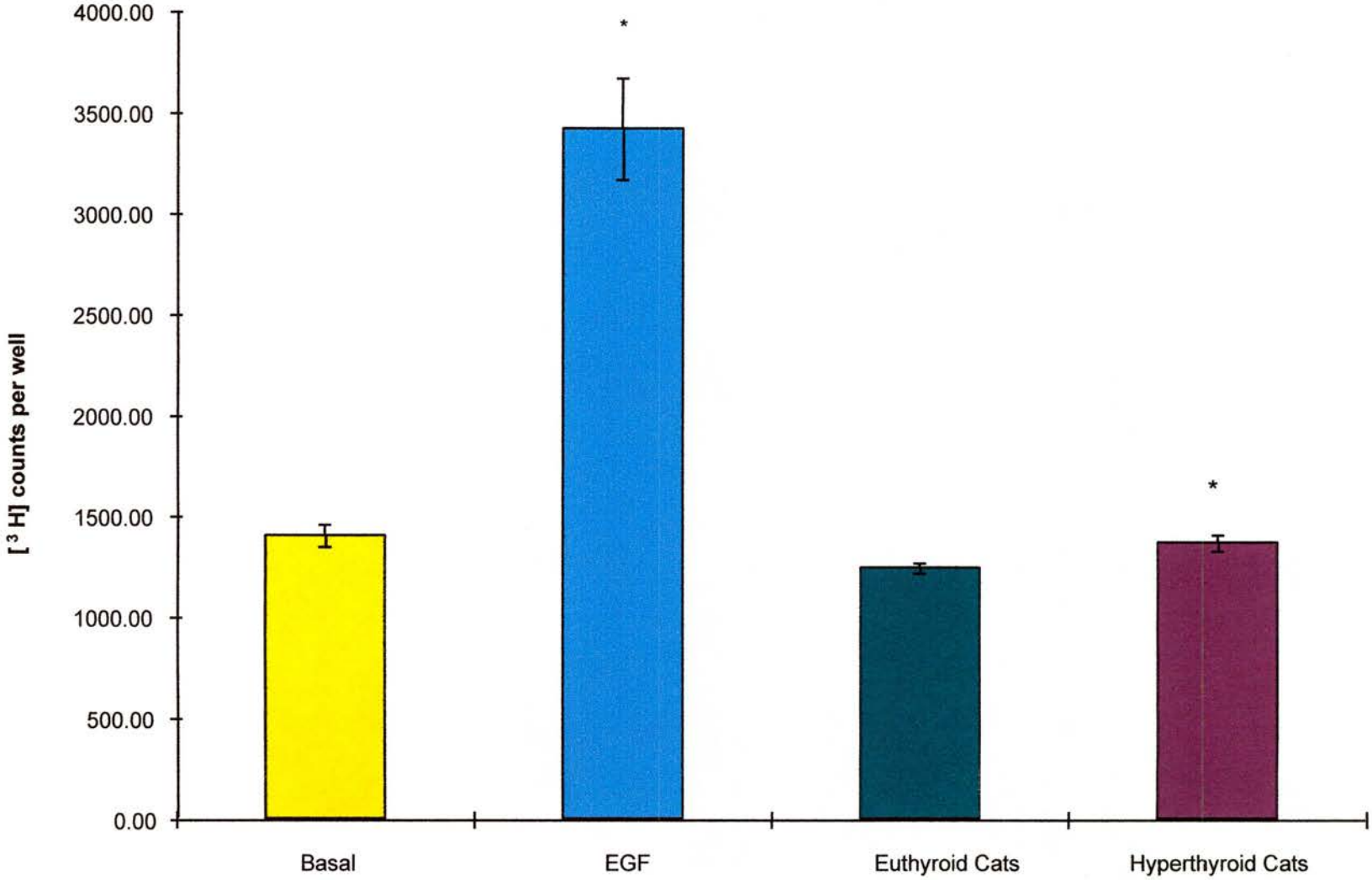


Figure 4.10 : Growth of feline thyrocytes (FT50) in response to purified IgG (1mg/ml) Comparison between basal cell growth, epidermal growth factor (EGF) (1ng/ml) and cells stimulated by IgG from euthyroid and hyperthyroid cats. Results shown are the mean of all cats in a group ± SEM. $p<0.05$*.

4.04 DISCUSSION

There is general agreement that more than one factor and second messenger pathway are responsible for expressing the full proliferation potential of thyrocytes (Tramontano and Villone, 1994). It is also accepted that without an initial rise in intracellular cAMP, thyrocytes cannot enter S phase and therefore cell division cannot occur (Tramontano and Villone, 1994). Furthermore, this increase in cAMP must be degraded before growth effects are seen. This fluctuation of cAMP is thought to be the trigger for cell proliferation (Tramontano and Villone, 1994). Additionally, "cross-talk" between second messenger systems is now regarded as an important mechanism in the control of growth and function of cells, particularly thyrocytes (Burrow and Eggo, 1994; Tramontano and Villone, 1994).

Methods used to detect growth, including the technique of [^{3}H]-TDr incorporation used here have been questioned. However, with this technique, it is usually false positive results that are problematic.

There are a number of factors that can give rise to criticisms in the investigation of the presence of growth stimulating immunoglobulins. In the present study, attempts were made to try to eliminate these by using preferred methods of immunoglobulin preparation and growth assays. Purification of IgG using ammonium sulphate precipitation reduces TSH receptor binding of IgG compared to the use of diethylaminoethyl or protein A affinity chromatography (Brown, 1995). Furthermore, protein A affinity purification results in a more pure preparation of IgG compared to that obtained with DEAE ion exchange chromatography (Wardle, Weetman, Mitchell *et al.,* 1993). The method of purification of IgG in the investigation of autoantibodies is sometimes critical in the evaluation of a disease *in vitro.* Wardle, Weetman, Mitchell *et al.* (1993), investigating adrenocorticotropic hormone receptor-blocking immunoglobulins in patients with Addison's disease, demonstrated that IgG purified by DEAE cellulose appeared to show receptor blocking antibodies in 41% of patients, whereas protein A affinity purification did so in only 2% of patients leading to the conclusion that this receptor antibody was in fact a contaminant and not IgG.

Other difficulties in comparing results from growth stimulating experiments is the cell type used and the conditions employed. FRTL-5 cells produce variable effects through widely divergent growth characteristics between strains (Brown, 1995). A report of IgG-induced growth in FRTL-5 cells in patients with endemic and non-endemic goitre could only demonstrate this effect in the presence of 50mU/ml bTSH (Wilders-Truschnig, Drexhage, Leb *et al.,* 1990). Brown, Keating,

Livingstone *et al.* (1992) could demonstrate a growth effect in cats with hyperthyroidism without the addition of TSH. Ultrasensitive cytochemical assays used to detect growth (the Feulgen reaction) do not require the addition of TSH to demonstrate growth effects (Brown, 1995) but have not been confirmed to be accurate indicators of growth *in vitro*. No growth assays described to date eliminate all error. In Brown's review on growth stimulating immunoglobulins (Brown, 1995), she concludes that one of the most important steps in determining growth effects in goitrous diseases is to eliminate species differences.

The results presented here have been unable to demonstrate any significant biological effect of bTSH on growth in normal or adenomatous feline thyrocytes at doses ranging from 0.001mU/ml to 500mU/ml. A growth response was detected in many thyrocyte preparations at concentrations of bTSH of 1000mU/ml. However it is likely that other growth factors which contaminate this pituitary extract of bTSH may be responsible for the growth response observed at this high concentration. Although it is generally accepted that, both *in vitro* and *in vivo*, TSH acts as a mitogen for thyrocytes of a number of species (dogs, rats and humans), it has not been demonstrated to do so in others (pigs, cattle, sheep) (Dumont, Maenhaunt, Pirson *et al.,* 1991). In species where TSH acts as a mitogen, the growth effect is reproduced by agents that enhance cAMP accumulation in cells (Vassart and Dumont, 1992). A lack of growth in response to TSH may reflect the inherent growth characteristics of the species studied, species specificity between TSH used and the species studied, or the cell culture environment (e.g. lack of specific growth factors required to enhance the TSH effect). Previous cell culture environment may also have permanent effects on the growth characteristics of cells. This is particularly true of exposure of thyrocytes to iodine (Dumont, 1989), which can lead to inhibition of growth *in vitro*. Passage number may also affect growth: FRTL-5 cells may lose their TSH responsiveness with increased passage number, whilst human thyrocytes sometimes show increased TSH responsiveness, and decreased EGF responsiveness, with increased passage number (Goretzki, West, Koob *et al.,* 1987). The fact that many investigators have demonstrated that TSH is a mitogen for thryocytes *in vitro* , and that human patients with thyroid carcinoma have increased longevity if their endogenous TSH is suppressed by supplementation with thyroid hormones, leads to the conclusion that TSH is important for growth of thyrocytes (Dumont, 1989).

In this study, feline thyrocytes, whether normal or adenomatous, grew in response to 10 to 200ng/ml EGF. This growth response observed is not unusual. EGF acts through a tyrosine kinase second messenger system and is a known growth factor of most mammalian cells (Nilsson, 1995). It is mitogenic for human, canine, porcine, ovine and bovine thyrocytes.

However, in some species such as dogs, EGF acts as a growth factor for thyrocytes only in the presence of insulin (Gartner, Tsavella, Bechter *et al.*, 1987). EGF growth stimulation is accompanied by a reversible loss of differentiated expression (Gartner, Tsavella, Bechter *et al.*, 1987). The present observations that IGF-1 also stimulated growth in normal and adenomatous feline thyrocytes at doses from 0.1 to 5ng/ml is also similar to that reported in other species. IGF-1 stimulates growth in porcine, ovine and human thyrocytes and FRTL-5 cells (Dumont, Maenhaut, Pison *et al.*, 1991). As bTSH did not produce growth in feline thyrocytes, EGF was used in these studies as a positive control in experiments where purified IgG was used as a stimulus for growth.

These results help to classify some of the *in vitro* growth characteristics of feline thyrocytes and demonstrate that [^{3}H]-TDr incorporation can be used to detect growth in this cell model. Although normal feline thyrocytes appeared to have increased [^{3}H]-TDr incorporation in response to EGF and IGF-1, it is difficult to compare absolute data between experiments. Generally, there appeared to be no inherent difference between the growth responses of normal or adenomatous thyrocytes to bTSH, EGF or IGF-1.

In this study, identical methods were used in an attempt to confirm the results described by Brown *et al.* (1992) who reported that purified IgG from some hyperthyroid cats caused significant growth compared to that from euthyroid cats in FRTL-5 cells, and that this effect was similar to that of 10mU/l bTSH. Brown's group showed that 0.5 to 1mg/ml (final concentration) IgG caused maximum growth of FRTL-5 cells. The present study also demonstrated growth stimulation in this cell line using purified IgG (1mg/ml) but this occurred with IgG from both euthyroid and hyperthyroid cats. Overall, there was a significantly greater growth response in IgG preparations from euthyroid cats compared to hyperthyroid cats ($p = 0.0258$), although this difference was small. This difference may be statistically significant but is not as great as the growth effect reported in this cell line by Brown *et al.* (1992), and is unlikely to be of biological significance. FRTL-5 cells are useful for detecting TGIs for a number of reasons. Their growth characteristics are well-defined and the population of cells is heterogenous. They do not, however, respond as primary cultures of thyrocytes do, as they have an absolute requirement for TSH and a complex hormonal mix in the culture medium.

When normal feline thyrocytes were used in the present study in an attempt to examine the growth effects of feline IgG, that derived from hyperthyroid cats significantly stimulated growth compared to that derived from euthyroid cats ($p = 0.0333$). Like the results in FRTL-5 cells, this

difference was statistically significant but is so small that it is unlikely to be of biological significance. Furthermore, neither IgG from euthyroid nor hyperthyroid cats stimulated growth when compared to basal ($p = 0.2620$ and 0.5508 respectively). Primary cell cultures are useful for determining the presence of TGIs to eliminate cross-species differences. The cell population, however, is not homogenous and the cells are not immortal. Hence, the isolated cells can only be used for 1 to 2 weeks, and ideally within the first 1 to 2 passages for growth experiments (Ambesi-Impiombata and Villone, 1987). Each preparation from a different individual will undoubtedly share different growth characteristics.

There are a number of reasons that may explain why purified IgG from feline serum causes growth of FRTL-5 cells, whilst not stimulating growth of feline thyrocytes *in vitro*. The first is the possibility of a contaminating factor in the IgG preparation which was able to stimulate the growth of FRTL-5 cells but not feline thyrocytes. Crude IgG preparations (from PEG or ammonium sulphate precipitation) can sometimes contain EGF which stimulates growth, although the further purification with DEAE sepharose or Staphlococcal protein A should eliminate this contamination (Gartner, Tsavella, Bechtner *et al.*, 1987). Although the IgG preparations used in the present study were shown to be pure by SDS-PAGE, they may have been contaminated with low molecular weight growth factors that would not appear on the SDS-PAGE gel. However, it is unlikely that EGF would be the responsible growth factor for FRTL-5 cells in either the present or Brown's experiments, as these cells do not grow in response to EGF (Gartner, Tsavella, Bechtner *et al.*, 1987). Additionally, the feline thyrocytes in this study did not grow significantly from basal in response to the feline IgG preparation. The fact that the growth effect observed in FRTL-5 cells with purified IgG did not stimulate growth in feline thyrocytes suggests that the growth effect observed may be due to an unidentified contaminant. Furthermore, the growth demonstrated in FRTL-5 cells in response to IgG preparations from both euthyroid and hyperthyroid cats further increases this suspicion. As the growth effect was not significantly different from basal in feline thyrocytes, it is difficult to imagine that IgG has a role in the pathogenesis of feline hyperthyroidism.

Brown *et al.* (1992) concluded that some hyperthyroid cats had an antibody that bound to the TSH receptor and induced growth. They were, however, unable to demonstrate activation of the cAMP pathway which is thought to be a prelude to growth in thyrocytes. It is possible that these TGIs act to directly stimulate the PIP pathway to induce growth. Receptor antibodies are able to act with discreet units of the TSH receptor and it is possible that TGIs could preferentially activate the PI pathway. The exact mechanism(s) for selective triggering of pathways is

unknown. However, as cAMP and its analogues are such common inducers of growth in thyrocytes from many species, it is difficult to imagine that in feline thyrocytes, cAMP stimulation would not be a prerequisite for growth in the pathogenesis of hyperthyroidism. TSH is known to induce PI stimulation only in high concentrations. As the results in Chapter 5 indicate, the results of this study were also unable to demonstrate increased cAMP accumulation in a number of cell types (normal and adenomatous feline thyrocytes and CHO cells), whether sera or purified IgG was used.

Another possible explanation for the difference between these results and those of Brown *et al.* (1992) may be the population of cats studied. Goitrogenic antibodies may appear early in the disease process (described in detail in Chapter 5) and may actually be produced within the thyroid by intrathyroidal lymphocytes (Schatz, Ludwig, Wiss *et al.,* 1987).

The existence of TGIs in human thyroid disease other than Graves' disease has caused much debate. In 1978, Brown and others demonstrated that purified IgG from patients with Graves' diseases, Hashimotos thyroiditis and toxic and non-toxic nodular goitre could inhibit binding of radiolabelled TSH to its receptor. Although no assessment of the growth potential of these IgGs was undertaken at this time, it began the debate as to the significance of TGIs in forms of thyrotoxicosis not previously thought to be immune-mediated. Drexhage, Bottazzo, Doniach *et al.* (1980) and later Smyth, McMullen, Grubeck-Loebenstein *et al.* (1986) reported that 60 to 70% of patients with non-endemic sporadic goitre had serum TGIs.

In the present study, feline IgG was used to test for growth effects in feline thyrocytes and none were found. In the present study, the differences found between the growth effect from basal in FRTL-5 cells and feline thyrocytes may be due to the different medium used to supplement FRTL-5 cells. FRTL-5 cells are supplemented with '5H' medium in these experiments and feline IgG may require additional growth factors (such as insulin, somatostatin, transferrin) to stimulate growth in feline thyrocytes. The most important point is that there was no biological difference between purified IgG from euthyroid and hyperthyroid cats in either cell system. Had a biologically significant growth effect occurred in either FRTL-5 cells or feline thyrocytes cell counting would have been used to confirm it. The lack of a growth effect in feline thyrocytes in response to purified IgG from hyperthyroid cats in these experiments, and the lack of adenyl cyclase activation in Brown's experiments or the present experiments (Chapter 5), all strongly suggest that a humoral stimulus is not involved in the pathogenesis of feline hyperthyroidism.

5.00 SECOND MESSENGER PATHWAY ACTIVATION IN THE FELINE THYROCYTE AND THYROID STIMULATING IMMUNOGLOBULINS

5.01 INTRODUCTION

Two major signalling pathways, (adenyl cyclase and phospholipase C) produce the majority of physiological and biochemical effects in the thyrocyte of many species. The TSHR is coupled to both systems and the stimulation of one or more of the signalling pathways is both species and effector dependent. In Graves' disease, a range of antibodies directed against the TSH receptor are produced. Some are able to displace TSH binding from the TSHR *in vitro* (TBIIs), some stimulate cAMP accumulation in various cell types (primary cultures of thyrocytes, FRTL-5, CHO) (TSIs), whilst others stimulate growth (TGIs).

The presence of thyrotropin receptor antibodies (TRABs) can be determined by a number of methods. These include investigating the second messenger response of cells in culture, measurement of iodide uptake in FRTL-5 cells or using a competition assay to determine the displacement of binding of radiolabelled TSH from porcine thyrocyte membranes by TSHR antibodies: the classic 'TRABs' assay. The sensitivity and specificity of each technique varies. Graves' disease patients commonly have antibodies in serum that cause cAMP accumulation and stimulate iodine uptake. TBIIs are often also present (Kasagi, Hatabu, Tokuda *et al.,* 1988).

The degree by which sera stimulate such assay systems is dependent on the patient's TRABs titre and other factors such as type of sample (unpurified sera, crude immunoglobulin preparations, or purified IgG), the cell system (e.g. FRTL-5, JPO9) and the culture medium used (Hank's buffered saline [HBSS], NaCl-free HBSS). The methods of purification of IgG also vary widely and can influence the degree of stimulation in biological systems. Variations in methods for the purification of immunoglobulins have been previously discussed in Chapter 1.03.

A number of cell types have been used to demonstrate thyroid stimulating activity. Primary cell isolates as reporter cells were first used to investigate second messenger pathway activation with TRABs. Kasagi, Konishi, Iida *et al.* (1982), reported on cAMP production in human thyroid adenoma cells following exposure to serum from hyperthyroid and euthyroid (treated) Graves' disease patients. PEG precipitated immunoglobulin fractions in NaCl-free conditions were used in their assay. Rapoport, Filetti, Takai *et al.* (1982) also measured cAMP responses in human thyroid cells but Graves' immunoglobulins were prepared by ammonium sulphate precipitation in NaCl-free conditions.

More recently, the development of cell lines has allowed further investigations into the effects of TSIs. The ease of obtaining and maintaining cell lines has made them a popular cell system. Such cell lines include the transformed rat thyrocyte cell line (FRTL-5) and the Chinese hamster ovary cell line transfected with human TSHR (JPO9).

Using crude PEG-precipitated immunoglobulin fractions in salt-free conditions, the cAMP production, TSH binding inhibitory characteristics and stimulation of radioiodine uptake in FRTL-5 cells in response to hyperthyroid and euthyroid Graves' disease patients have been previously reported (Kasagi, Konishi, Iida *et al*, 1988; Hidaka, Kasagi, Takeuchi *et al*, 1994).

JPO9 cells have also been widely used to assess the presence of cAMP-stimulating immunoglobulins (Michelangeli, Munro, Poon *et al.,* 1994). The main advantages of these cells are that they have simpler growth requirements in comparison to FRTL-5 cells, do not require withdrawal of TSH prior to the assay and respond to unpurified sera from TRABs positive patients compared to FRTL-5 cells (Michelangeli, Munro, Poon *et al.,* 1994). For these reasons, JPO9 cells may be a preferable bioassay system compared to FRTL-5 cells for detecting cAMP dependent thyroid stimulators.

The sensitivity of cell lines to cAMP stimulation in response to TRABs antibodies depends greatly on the isotonicity of the medium used. It appears that NaCl-free medium improves sensitivity of TRABs detection in FRTL-5 cells (Kasagi, Konishi, Arai *et al.,* 1986; Kasagi, Konishi, Iida *et al.,* 1987; Hikada, Kasagi, Takeuchi *et al.,* 1994), porcine thyrocytes (Kasagi, Hidaka, Hatabu *et al.,* 1989), human thyroid adenoma cells (Kasagi, Konishi, Iida *et al.,* 1982) and human thyroid cells (Rapoport, Filetti, Takai *et al.,* 1982). The mechanisms for this are reported to include increased ligand binding, increased membrane permeability and post-receptor mechanisms that prevent TSHR damage and promotion of intracellular cAMP production (Kasagi, Hidaka, Hatuba *et al.,* 1989).

Cell conditions also affect cAMP response. FRTL-5 cells that are seeded into wells in 5H medium initially plate down, but do not grow, and show poor cAMP responses compared to those initially seeded into wells with 6H medium and deprived of TSH over a 5 day period (Vitti, Rotella, Valente *et al.,* 1983). Cell density is also important, the ideal number of cells for second messenger experiments being 5×10^4 to 5×10^5 cells per well in 24 well plates (Vitti, Rotella, Valente *et al.,* 1983).

Whilst immortalised cell lines such as FRTL-5 and JPO9 cells may provide a useful model to study the presence of TSIs in the cat, they may be unsatisfactory because of possible species differences in the specificity and sensitivity of the TSHR to feline TSIs. The use of primary cultures of feline thyrocytes may provide a more reliable and sensitive means of studying the presence of TSIs in the serum of thyrotoxic cats. Studying second messenger systems in the feline thyrocyte in addition to CHO cells not only allows us to determine the sensitivity of cAMP and PIP pathways to TSH in the feline thyrocyte, but may also allow an *in vitro* system for diagnosis or subclassification of feline thyrotoxicosis if TSIs are important in the pathogenesis of this condition.

The aims of this study were therefore to :

- determine if bTSH produces a consistent cAMP response in adenomatous feline thyrocytes (FNG) and normal feline thyrocytes (FT) as would be expected from studies in other species

- study the phosphoinositol cascade in feline thyrocytes in response to bTSH and ATP

- to assess the cAMP response of FNG, FT, transfected (JPO9) and untransfected (JPO2) CHO cells, to unpurified pooled sera, unpurified non-pooled sera and purified IgG from biochemically euthyroid and hyperthyroid cats, using bTSH and TRABs positive human serum as positive controls and serum from biochemically euthyroid human blood donors as negative controls.

- to assay for TBIIs in sera and purified IgG preparations from hyperthyroid cats.

5.02 MATERIALS AND METHODS

a) STIMULATION OF cAMP BY bTSH, SERUM, AND PURIFIED IgG IN FELINE THYROCYTES AND CHO CELLS (JPO9 & JPO2).

Feline thyrocytes were isolated as described in Chapter 2.037. All cell types (feline thyrocytes and JPO9) were gently trypsinised from flasks with 1x trypsin/EDTA at 37°C and plated out at a density of $5x10^5$ cells per ml into 24 well plates. For feline thyrocytes (FNG & FT), a passage number of up to two was used. JPO9 and JPO2 cells were all below passage number 10. It is known that FRTL-5 cells lose their sensitivity to TSH with increasing passage number and this may also be true in other thyrocytes (such as FNG & FT) or cells expressing TSH receptors (JPO9). After 24 hours, the cells were washed twice with 0.5ml of EBSS. For experiments involving serum, 450µl of respective medium (RPMI for feline thyrocytes, Hams F-12 for CHO cells) was then added to each well in the presence of 800µm IBMX (potent phosphodiesterase inhibitor to prevent breakdown of cAMP), and the cells incubated for 15 minutes prior to the addition of test serum to their final concentration (1 to 5% v/v). Initially, pooled serum was used to determine if there was a particular time point that showed significant cAMP accumulation. For experiments involving purified IgG (1mg/ml), NaCl-free HBSS was used instead of medium. An IgG concentration of 1mg/ml was chosen as this concentration has previously been shown to be optimal for cAMP (Vitti, Rotella, Valente *et al.,* 1983) and growth assays (Vitti, Elisei, Tonachera *et al.,* 1993) in human studies and feline studies (Brown, Keating, Livingstone *et al.,* 1992). For a positive control, bTSH (500mU/l final concentration) was used to assess cAMP stimulation unless otherwise stated.

At the end of incubation, the medium was removed, placed in glass tubes and immediately acidified by the addition of 5µl of 20%v/v acetic acid per 500µl of medium. Samples were then kept at 4°C until the experiment was completed. Samples were acetylated with the same batch of acetylation mix (triethylamine: acetic anhydride, 2:1) prior to assay for cAMP using an in-house RIA as described in Chapter 2.042.

After incubation, the intact cells were gently washed three times with EBSS and 500µl of 1M NaOH was added to each well. The cells were left to solubilise overnight at 5°C and protein concentrations determined as described in Chapter 2.036. The cAMP concentration in the culture medium was then corrected for protein concentration.

b) PHOSPHOINOSITOL CASCADE ACTIVATION IN FELINE THYROCYTES IN RESPONSE TO bTSH AND ATP.

Feline thyrocytes (FT) were gently trypsinised from flasks with 1x trypsin/EDTA and plated out at a density of $5x10^5$ cells per ml into 24 well plates. After 24 hours, the medium was removed and replaced with 500µl of F-10 medium (low inositol) containing 5% NCS to allow the incorporation of tritiated inositol. After 48 hours, 0.37MBq/ml of tritiated inositol was added to each well. The rest of the assay was performed as described in Chapter 2.044 with ATP (10^{-4} final concentration) and TSH (10mU/l final concentration) additions made in 50µl medium.

c) THYROTROPIN RECEPTOR ANTIBODY (TRABS) ASSAY

A commercial assay (TRABs Assay, RSR Ltd, Cardiff) was used to establish the presence of TRABs in the serum and in purified immunoglobulin preparations from hyperthyroid and euthyroid cats. The method used is described in Chapter 2.043. The TRABs titre was used to determine

the percentage displacement of TSH compared to non-specific binding (NSB) by the following equation as per the method used by Brown, Keating, Livingstone *et al.*, 1992:

$$1 - \frac{\%\text{ bound in test - NSB}}{\%\text{ bound in 0 standard - NSB}} \times 100$$

When purified IgG was substituted for serum samples from the same cats in this assay, the TBII ranged from 93.5 to 100% in both euthyroid and hyperthyroid cats (data not shown). This was an artefact of the assay due to marked change of the matrix from serum to low salt HBSS (in which the immunoglobulins were solubilised). It was therefore necessary to dilute the assay standards and the IgG samples 1:2 with TRABs negative human serum to prevent this displacement of TSH binding.

d) STATISTICAL ANALYSIS

The ANOVA test was used to determine statistical difference between time points and the Mann Whitney U test was used to test for differences between groups.

5.03 RESULTS

a) cAMP ACCUMULATION IN MEDIUM WITH FELINE THYROCYTES IN RESPONSE TO bTSH

Both normal (n=1) and adenomatous cells (n=3) showed an approximately linear response to 500mU/l bTSH over 720 minutes (12 hours), thereafter the rate of cAMP accumulation increased over the next 12 hours. The cAMP concentrations were significantly elevated from basal ($p<0.05$) at all time points from 30 minutes to 1440 minutes (24 hours) ($p< 0.05$) An example of the cAMP response in a normal thyrocyte preparation and adenomatous thyrocyte preparation is illustrated in Figs. 5.01 and 5.02 respectively.

b) PHOSPHOINOSITOL CASCADE ACTIVATION IN NORMAL FELINE THYROCYTES IN RESPONSE TO bTSH AND ADENOSINE TRIPHOSPHATE (ATP)

The phosphoinositol cascade in normal feline thyrocytes was examined in response to 10mU/ml bTSH and ATP (10^{-4}). Activation of the PI pathway in response to TSH and ATP was observed in feline thyrocytes at 120 minutes. ATP induced a more consistent and greater response than TSH (Fig. 5.03).

c) cAMP ACCUMULATION IN FELINE THYROCYTES IN RESPONSE TO POOLED SERUM FROM EUTHYROID AND HYPERTHYROID CATS AT CONCENTRATIONS FROM 1 to 5% v/v FINAL CONCENTRATION.

i) Nodular goitre thyrocyte preparations

Two thyrocyte preparations from cats with thyrotoxicosis were tested for cAMP accumulation in response to pooled sera from thyrotoxic and euthyroid cats at concentrations of 1%, 2%, or 5% v/v final concentration in the medium.

Feline nodular goitre preparation 15 (FNG15)

At 30 minutes incubation, pooled euthyroid cat serum at 2% and 5% significantly stimulated cAMP accumulation compared to basal (no additions) (p=0.004 and 0.0100 respectively). Pooled hyperthyroid serum at 2% and 5% also stimulated cAMP accumulation at 30 minutes (p= 0.0040 and 0.0470 respectively) and at 240 minutes (p= 0.0000 and 0.0100 respectively). At 1440 minutes incubation, only 2% pooled thyrotoxic cat serum showed a significantly increased cAMP response compared to basal (p=0.0210). All increases in cAMP accumulations from serum were eight to 10 fold less than 500mU/l bTSH (Fig. 5.04) (bTSH results previously displayed in Fig. 5.02).

Pooled hyperthyroid cat serum produced an enhanced cAMP response in this preparation at 240 minutes at concentrations of 2 and 5% (v/v) (p = 0.0180 and 0.0340 respectively), and at 2% (v/v) at 1440 minutes (p = 0.0460). No other time point demonstrated that pooled euthyroid or

hyperthyroid feline serum caused significant stimulation of cAMP over basal in this cell preparation.

Feline nodular goitre preparation (FNG 16)

Only 500mU/ml bTSH proved to significantly increase cAMP concentrations in FNG16 above basal, although bTSH produced an approximately four-fold lower response over 1440 minutes than in FNG15 (Figure 5.02). Neither euthyroid nor hyperthyroid cat serum caused any significant increase in cAMP accumulation from basal over 1440 minutes (Fig. 5.05). Only thyrotoxic cat serum at 1% (v/v) significantly increased cAMP accumulation in this preparation at 720 minutes ($p = 0.0460$). At no other time points or concentrations tested were there any significant differences between euthyroid and hyperthyroid cats in this preparation.

ii) Euthyroid cat thyrocyte preparation

Normal feline thyrocyte preparation (FT4)

Pooled euthyroid cat serum (2%) at 480 minutes increased cAMP accumulation significantly above basal ($p = 0.0090$) (Fig. 5.06). Pooled hyperthyroid serum (1%, 2% and 5%) at 480 minutes also significantly increased cAMP accumulation above basal ($p = 0.0450$, 0.0420 and 0.0190 respectively). No other time point demonstrated that pooled euthyroid or hyperthyroid feline serum produced a significant stimulation of cAMP over basal in this cell preparation.

Thus, significant elevations in cAMP accumulation produced by normal feline thyrocytes occurred with both euthyroid and thyrotoxic pooled cat sera. Hyperthyroid cat serum failed to induce an enhanced cAMP accumulation when compared to euthyroid cats in this normal cat thyrocyte

preparation at any time point or serum concentration tested (Figure 5.06). In fact, at one time point (30 minutes), euthyroid cat serum at 1% (v/v) increased cAMP accumulation more significantly than thyrotoxic cat serum ($p = 0.0250$).

d) cAMP ACCUMULATION IN FELINE THYROCYTES IN RESPONSE TO INDIVIDUAL SERUM SAMPLES FROM EUTHYROID AND HYPERTHYROID CATS, TRABS POSITIVE HUMAN GRAVES' DISEASE PATIENTS AND EUTHYROID BLOOD DONORS AT 5% v/v FINAL CONCENTRATION

cAMP response was measured in feline nodular goitre cells (FNG 43) for a 2 hour incubation period with either 5% v/v unpurified feline serum from euthyroid or thyrotoxic cats, serum from TRABs positive Graves' disease patients or negative human blood donors as positive and negative controls respectively. Serum samples were added in NaCl-free HBSS, to enhance the cAMP response.

Serum from six of 10 (60%) euthyroid cats, and five of 10 (50%) hyperthyroid cats showed a significant cAMP response compared to basal. Serum from five of five (100%) TRABs positive and zero of five (0%) TRABs negative euthyroid human blood donors showed a significant cAMP response compared to basal. When data were grouped, there was a significant stimulation from basal with 500mU/ml bTSH ($p = 0.0040$), euthyroid feline serum ($p = 0.0142$), thyrotoxic feline serum ($p = 0.0142$), TRABs positive human sera ($p = 0.0282$) and TRABs negative euthyroid human sera ($p = 0.0369$) (Fig 5.07).

There was no significant difference between the cAMP response using euthyroid and hyperthyroid cat serum ($p = 0.4963$)(Figure 5.08), however there was a significant difference

between sera from TRABs positive Graves' patients and TRABs negative euthyroid sera (p = 0.0081).

e) cAMP RESPONSE OF JPO9 AND JPO2 CELLS TO POOLED SERA FROM EUTHYROID AND HYPERTHYROID CATS AT 5% v/v FINAL CONCENTRATION

JPO9 cells failed to show any significant response to pooled (5% v/v final concentration) euthyroid (p = 0.5690, 0.5650, 0.1800, 0.5230, 0.754 and 0.4000 for times 30, 120, 240, 480, 720 and 1440 minutes respectively), or thyrotoxic feline serum (p = 0.3530, 0.0880, 0.2220, 0.6810, 0.5070 and 0.1500 for times 30, 120, 240, 480, 720 and 1440 minutes respectively). 500mU/ml bTSH showed a significant stimulation in these cells at 30, 120, 240, 480, 720 and 1440 minutes (p = 0.0200, 0.0120, 0.0125, 0.0010, 0.0010 and 0.0029 respectively) (Fig 5.09). bTSH cAMP stimulation is shown separately in Fig. 5.10.

In contrast, JPO2 (control cells, no TSH receptor) cells did not show any significant cAMP response to bTSH, euthyroid or hyperthyroid cat sera at any time point. (Fig. 5.11).

f) cAMP RESPONSE IN JPO9 CELLS TO SERA FROM INDIVIDUAL EUTHYROID AND HYPERTHYROID CATS, TRABs POSITIVE HUMAN GRAVES' DISEASE PATIENTS AND TRABs NEGATIVE EUTHYROID HUMAN BLOOD DONORS AT 5% v/v FINAL CONCENTRATION.

JPO9 cells showed a significant increase in cAMP accumulation following a 2 hour incubation with 500mU/ml bTSH (p = 0.028), and with sera from six out of 10 euthyroid cats (p = 0.0100,

0.0090, 0.0080, 0.0150, 0.0080, and 0.0030). No thyrotoxic cats showed any significant increase in cAMP accumulation although this was probably due to the poorer precision of the assays when sera from hyperthyroid cats were used compared to euthyroid cats. A TRABs positive Graves' patient showed a significant increase in cAMP accumulation ($p = 0.0270$), whereas the euthyroid blood donor sample showed no significant cAMP accumulation from basal (Fig 5.12). Overall, there was no significant difference in cAMP response between euthyroid and thyrotoxic cats ($p = 0.1124$) (Fig 5.13).

g) cAMP RESPONSE IN JPO9 CELLS TO PURIFIED IgG (1mg/ml) FROM INDIVIDUAL EUTHYROID AND HYPERTHYROID CATS, TRABS POSITIVE HUMAN GRAVES' DISEASE PATIENTS AND TRABs NEGATIVE EUTHYROID BLOOD DONORS.

JPO9 cells showed a significant increase in cAMP accumulation following a 2 hour incubation with 500mU/ml bTSH ($p = 0.0030$), eight of 10 euthyroid (range of $p = 0.0020$ to 0.0320) and four of 10 thyrotoxic cats (range of $p = 0.0170$ to 0.0280), compared to basal. The TRABs IgG preparation also showed significant increase in cAMP accumulation ($p=0.0020$) whilst the euthyroid blood donor IgG sample did not (Fig 5.14). Overall, euthyroid cat sera showed significant cAMP stimulation in these cells ($p=0.0142$) compared to basal, whilst that from hyperthyroid cats did not ($p=0.0759$). Overall, there was no significant difference between euthyroid and hyperthyroid cats ($p= 0.0757$) (Fig. 5.15).

h) THYROTROPIN RECEPTOR ANTIBODIES (TRABs) IN EUTHYROID AND HYPERTHYROID CATS.

A commercial assay TRABs assay was used to determine the percentage displacement of TSH from its receptor compared to the zero standard (TBII) as described in the methods section of this chapter. The serum and IgG preparations (1mg/ml IgG) from 10 euthyroid and 10 hyperthyroid cats were used in this study.

Significant displacement of binding of TSH from the thyrotropin receptor was detected in the sera of 10 euthyroid and 10 hyperthyroid cats. However, the two TRABs positive samples gave a greater displacement. Euthyroid TRABs negative human serum gave no displacement (Fig. 5.16). However, there was no statistically significant difference between the TBIIs in euthyroid or hyperthyroid cats ($p = 0.6776$). There was no significant difference between the TBIIs of euthyroid or hyperthyroid cat IgG preparations from these 10 euthyroid and 10 hyperthyroid cats ($p = 0.2413$) (Fig. 5.17).

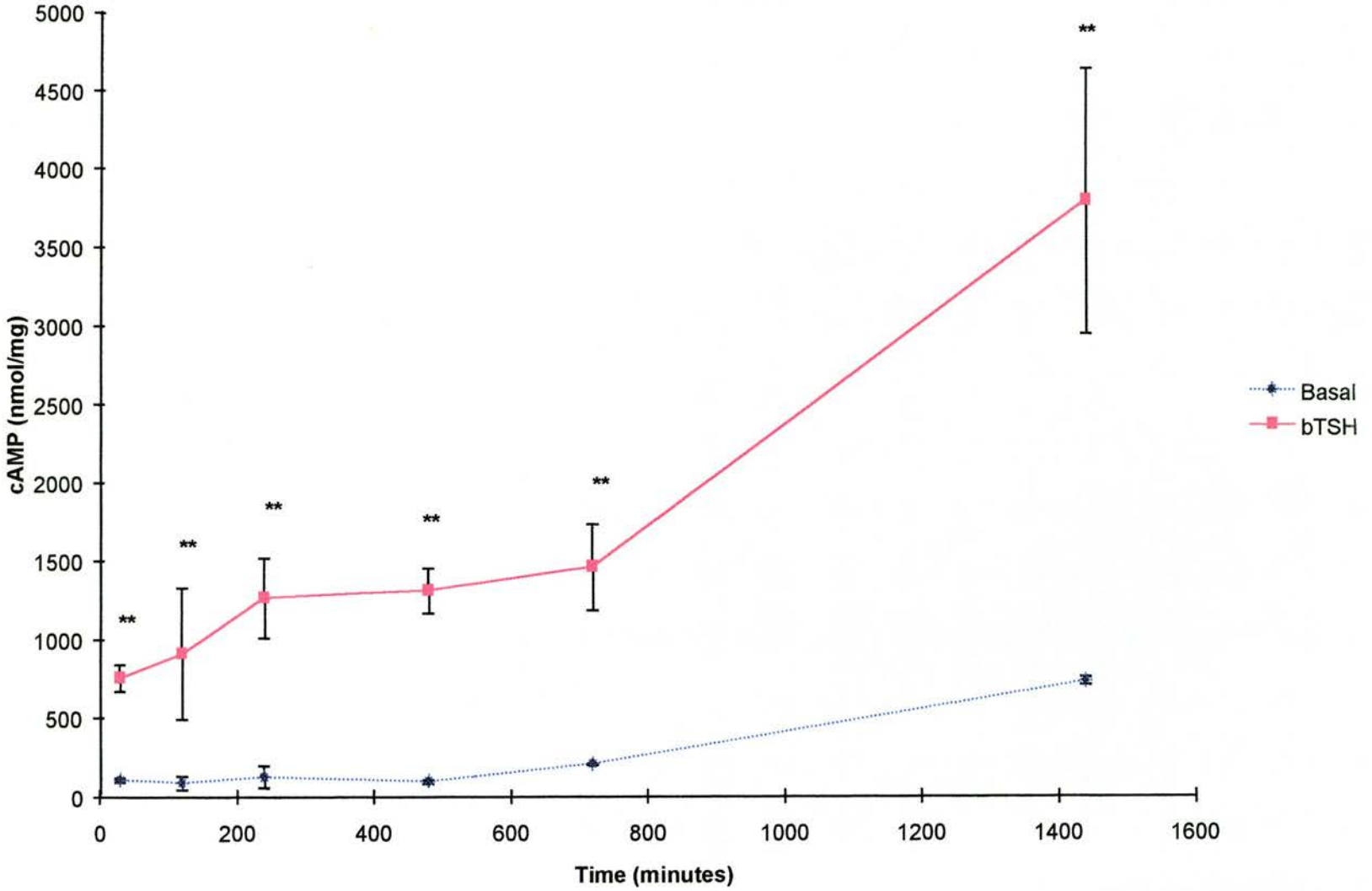


Figure 5.01: cAMP accumulation in normal feline thyrocyte preparation (FT4) following stimulation with bTSH (500mU/l) over 24 hours in the presence of 800uM IBMX. Results shown are those of the mean of triplicate wells ± SEM. $p<0.01$** from basal. bTSH, bovine TSH.

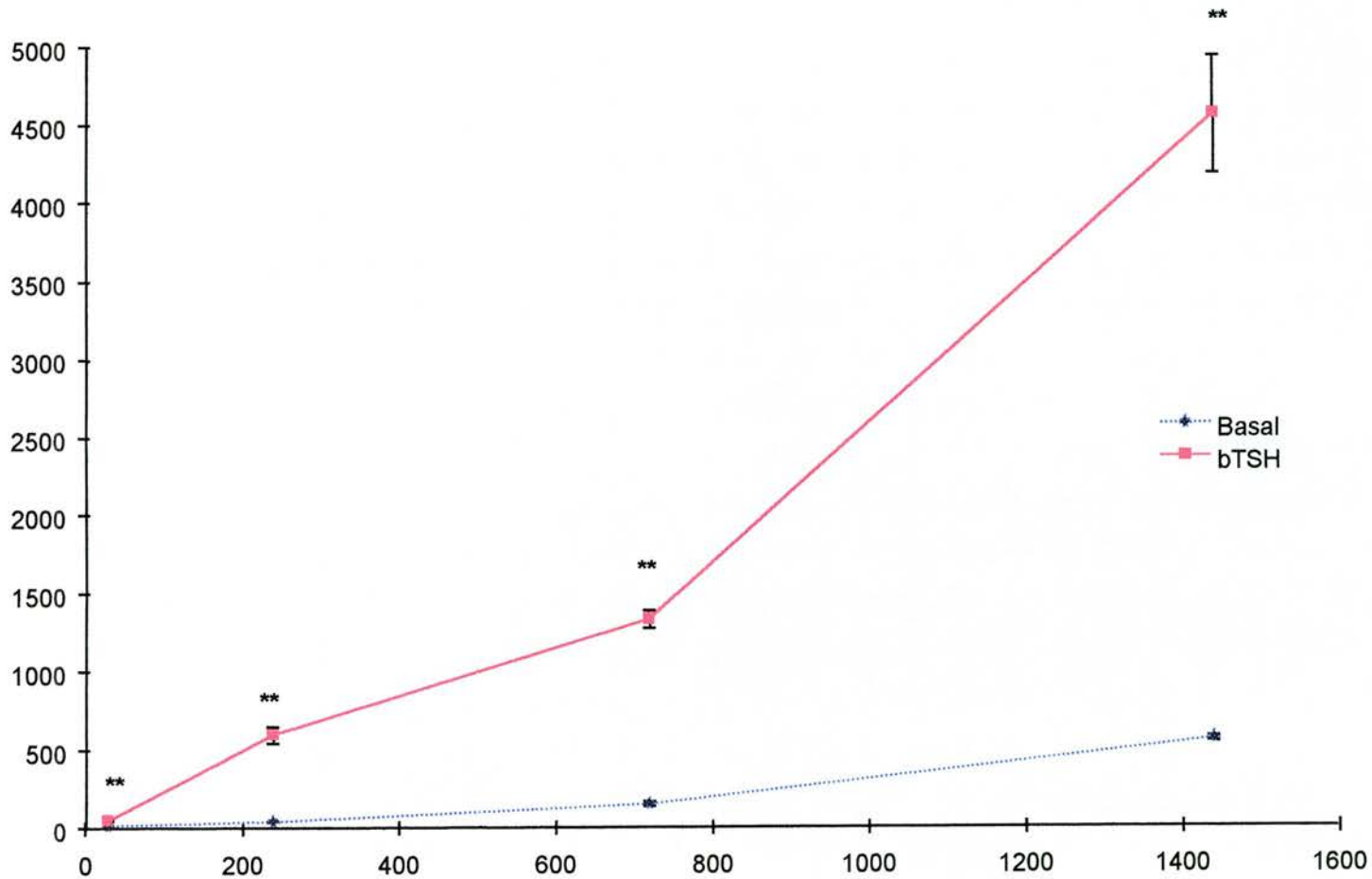


Figure 5.02: cAMP accumulation in adenomatous feline thyrocyte preparation (FNG15) following stimulation with bTSH (500mU/l) over 24 hours in the presence of 800uM IBMX. Results shown are those of the mean of triplicate wells ± SEM. $p<0.01$** over basal. bTSH, bovine TSH.

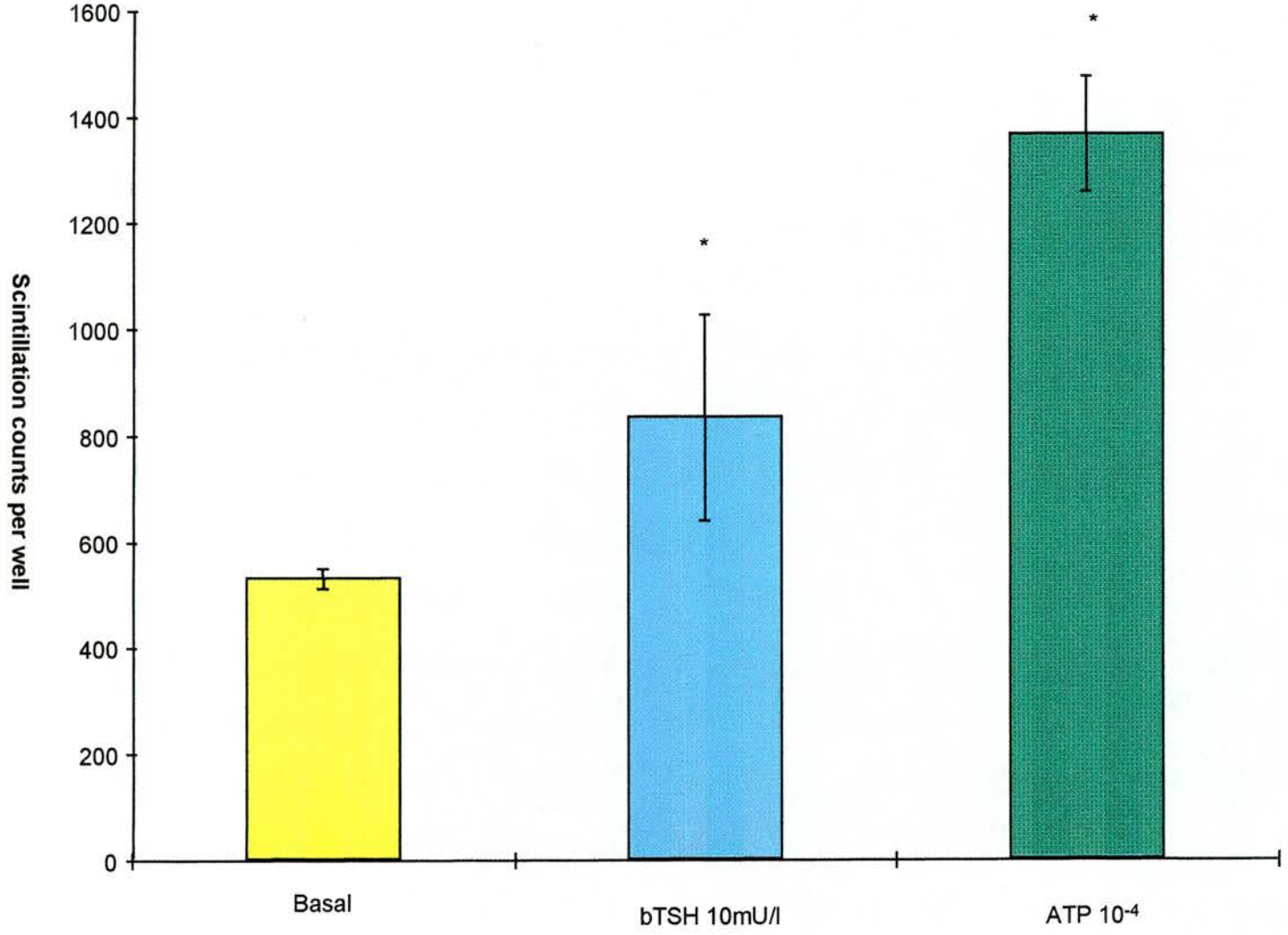


Figure 5.03: PI Accumulation In Normal Feline Thyrocyte Preparation (FT36) at 120 minutes. Data shown is mean of triplicate wells ± SEM. $p < 0.05$ * from basal. ATP, adenosine triphosphate; bTSH, bovine TSH.

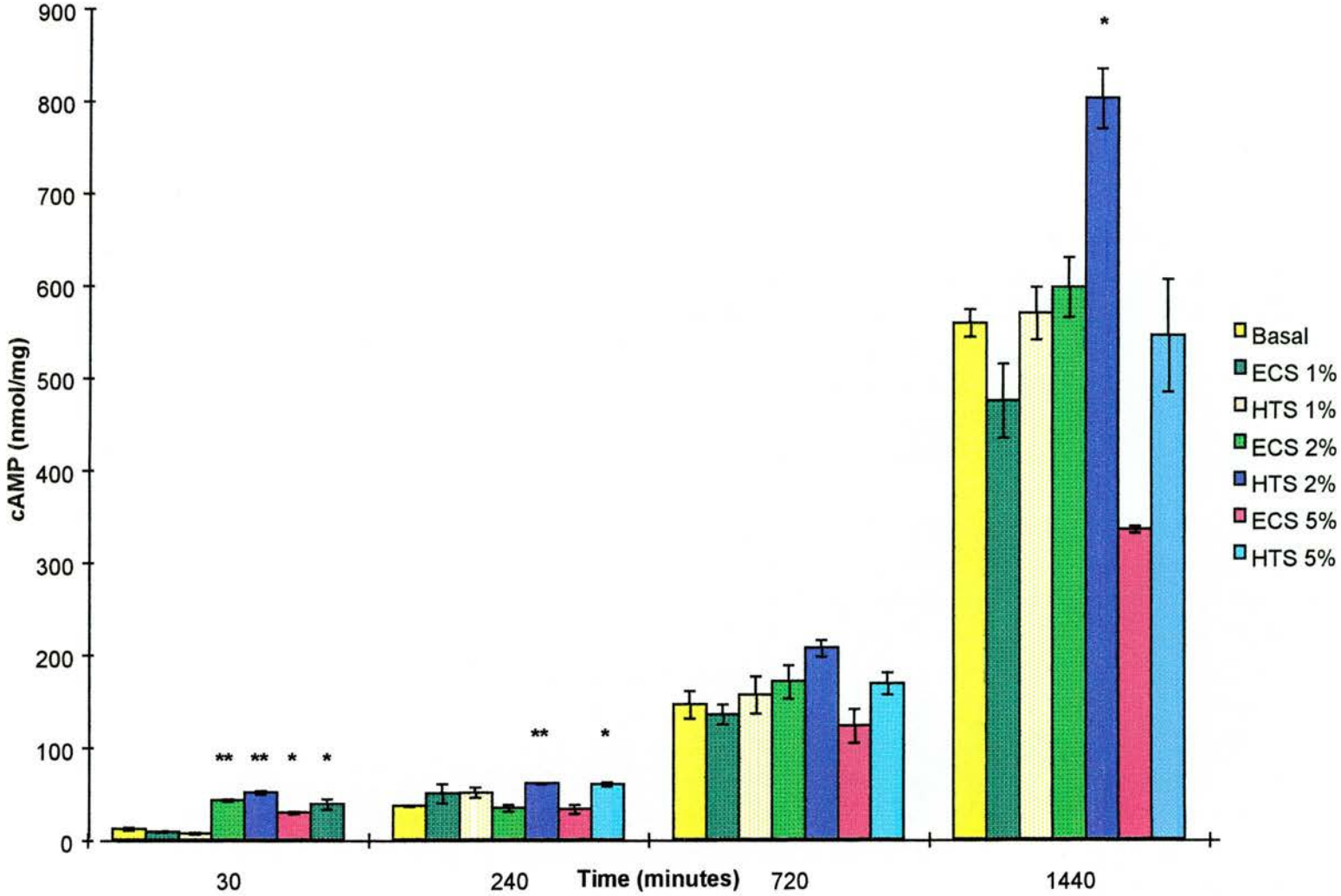


Figure 5.04: cAMP accumulation in an adenomatous feline thyrocyte preparation (FNG15) following stimulation with pooled sera from euthyroid and hyperthyroid cats at 1, 2 and 5% v/v final concentration over 1440 minutes (24 hours) in the presence of 800uM IBMX. Results shown are those of the mean of triplicate wells ± SEM. $p<0.05$* , $p<0.01$** from basal. ECS, euthyroid cat serum; HTS, hyperthyroid cat serum.

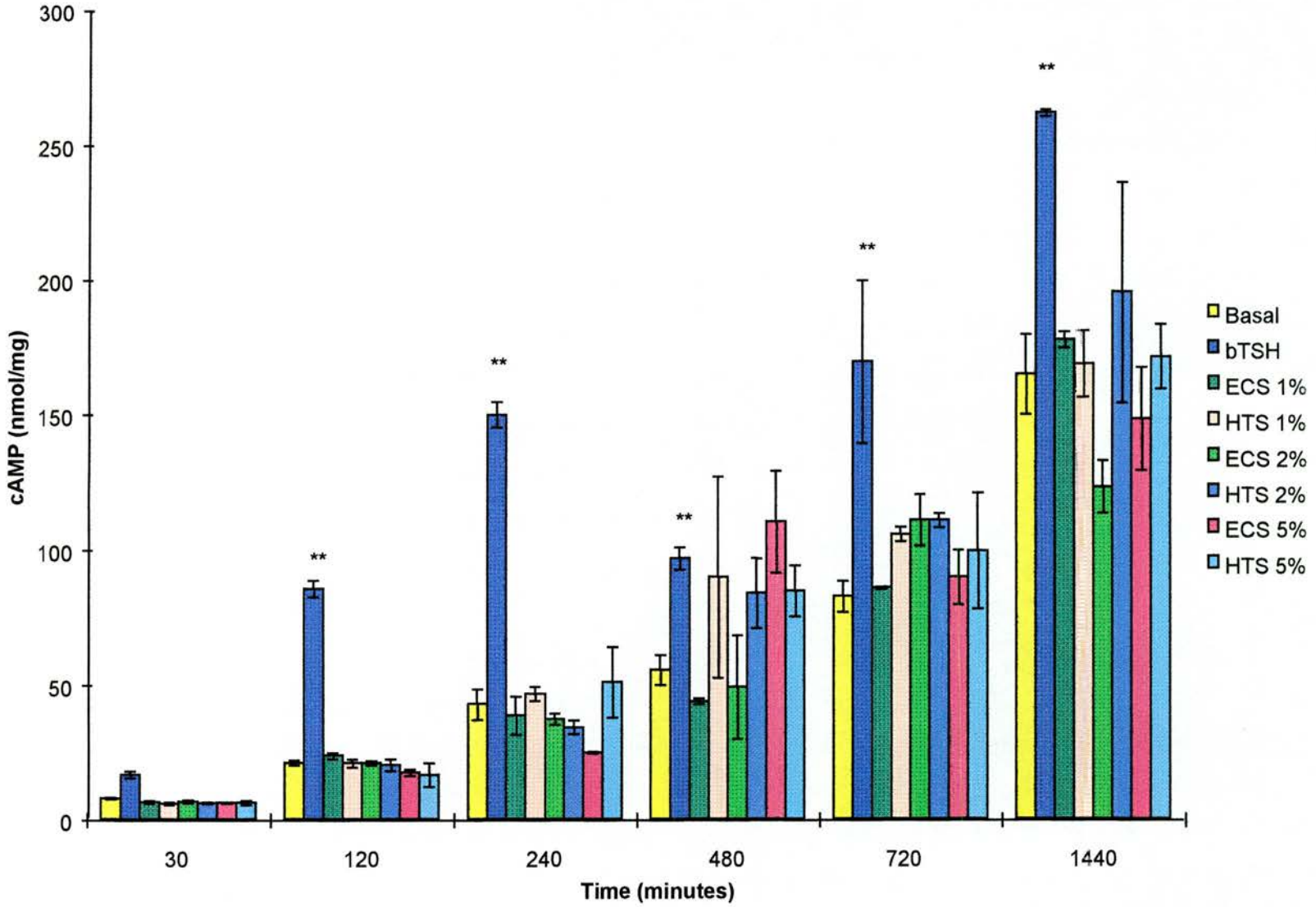


Figure 5.05: cAMP accumulation in an adenomatous feline thyrocyte preparation (FNG16) following stimulation with pooled sera from euthyroid and hyperthyroid cats at 1, 2 and 5% v/v final concentration over 24 hours in the presence of 800uM IBMX. Results shown are those of the mean of triplicate wells ± SEM. $p<0.01$** from basal. bTSH, bovine TSH; ECS; euthyroid cat serum; HTS, hyperthyroid cat serum.

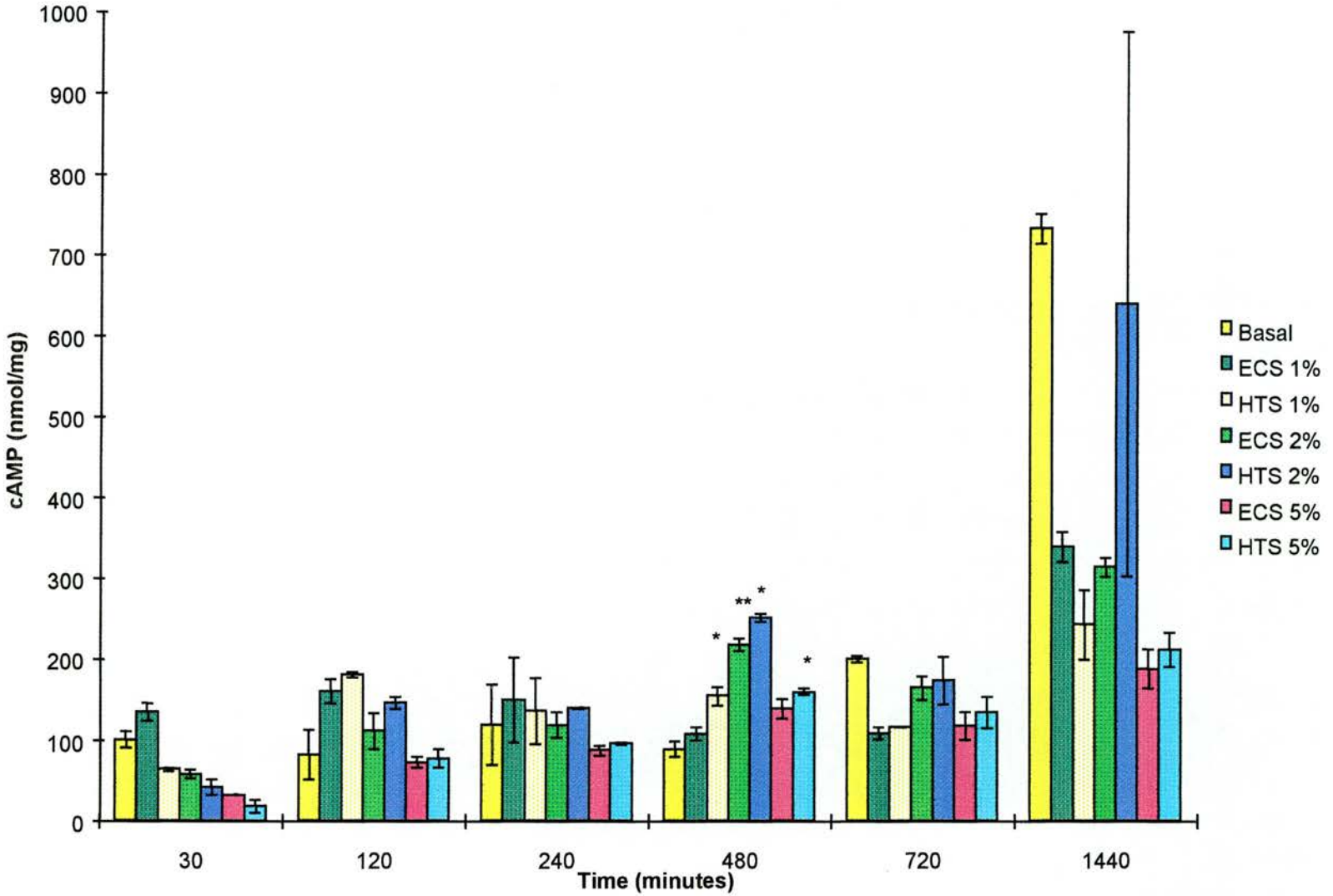


Figure 5.06: cAMP accumulation in a normal feline thyrocyte preparation (FT4) following stimulation with pooled sera from euthyroid and hyperthyroid cats at 1, 2 and 5% v/v final concentration over 1440 minutes (24 hours) in the presence of 800uM IBMX. Results shown are those of the mean of triplicate wells ± SEM. $p<0.05$*, $p<0.01$** from basal. ECS, euthyroid cat serum; HTS, hyperthyroid cat serum.

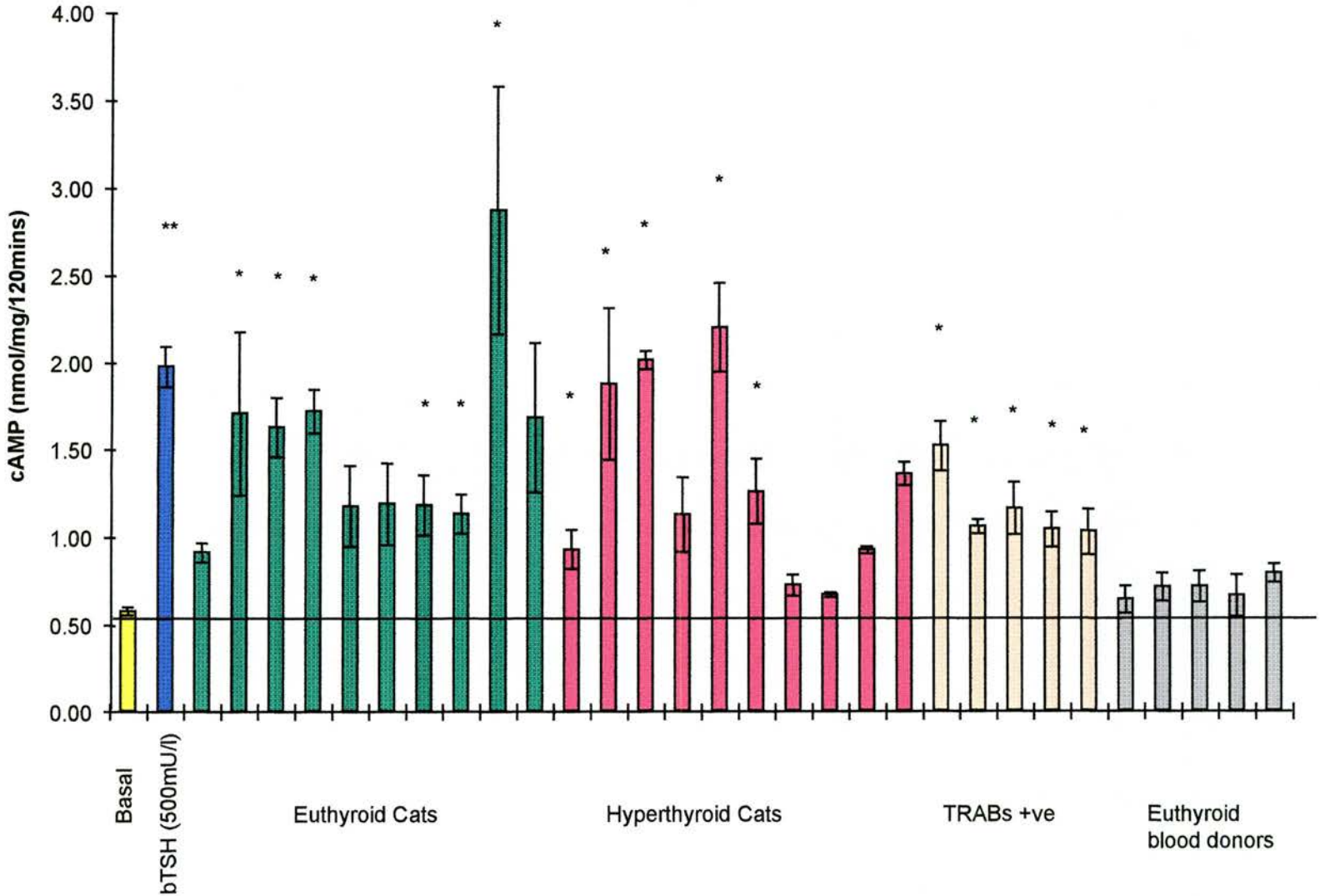


Figure 5.07: cAMP accumulation in an adenomatous feline thyrocyte preparation (FNG43) following stimulation with sera from euthyroid and hyperthyroid cats at 5% v/v final concentration over 120 minutes in the presence of 800uM IBMX. Results shown are those of the mean of triplicate wells ± SEM. $p<0.05$*,$p<0.01$** from basal. bTSH, bovine TSH; TRABs, thyrotropin receptor antibody positive.

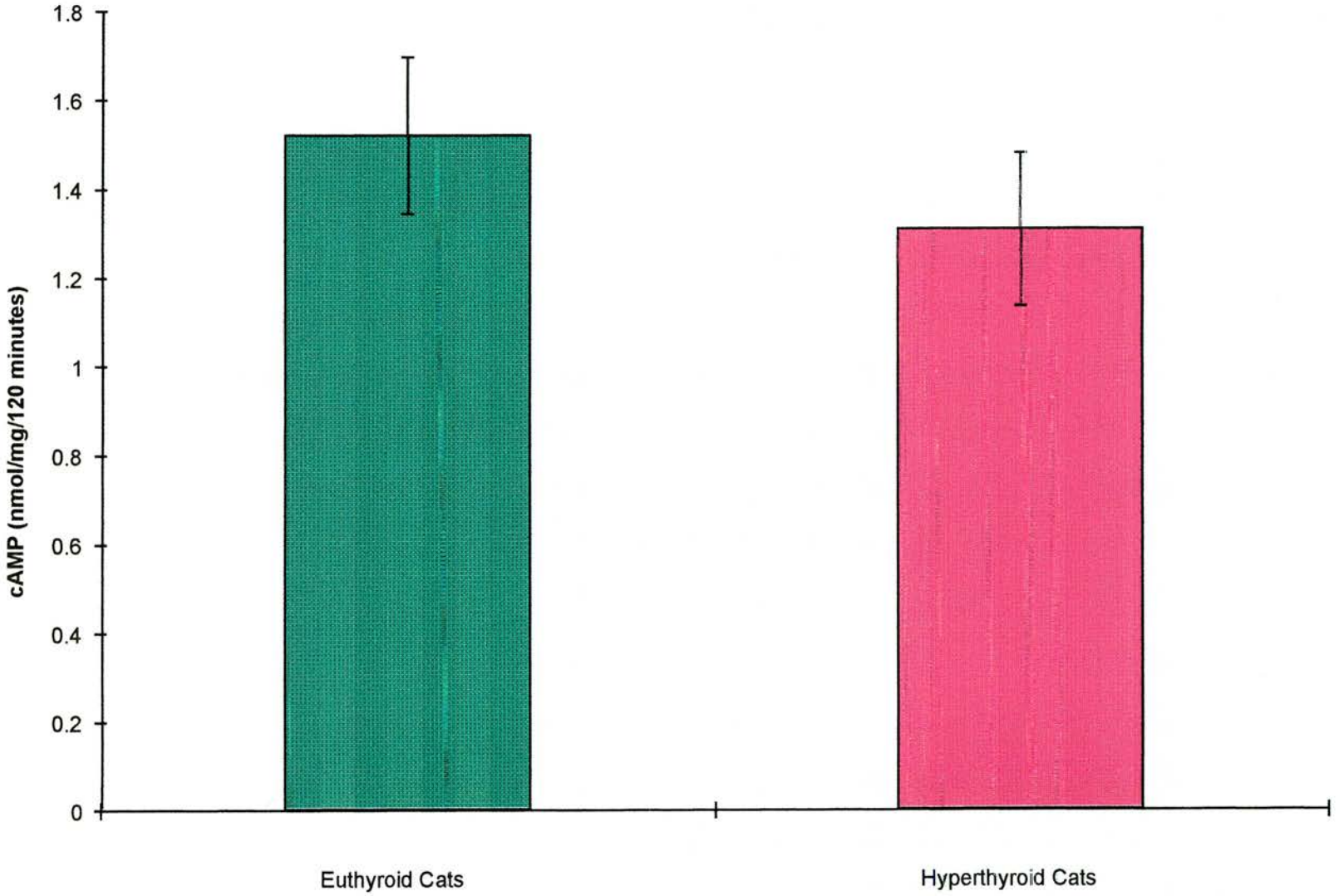


Figure 5.08: cAMP accumulation in an adenomatous feline thyrocyte preparation (FNG43) following stimulation with sera from euthyroid and hyperthyroid cats at 5% v/v final concentration over 120 minutes in the presence of 800uM IBMX. Comparison between euthyroid and hyperthyroid cats (n=10 for each group). Results shown are those of the mean of triplicate wells ± SEM.

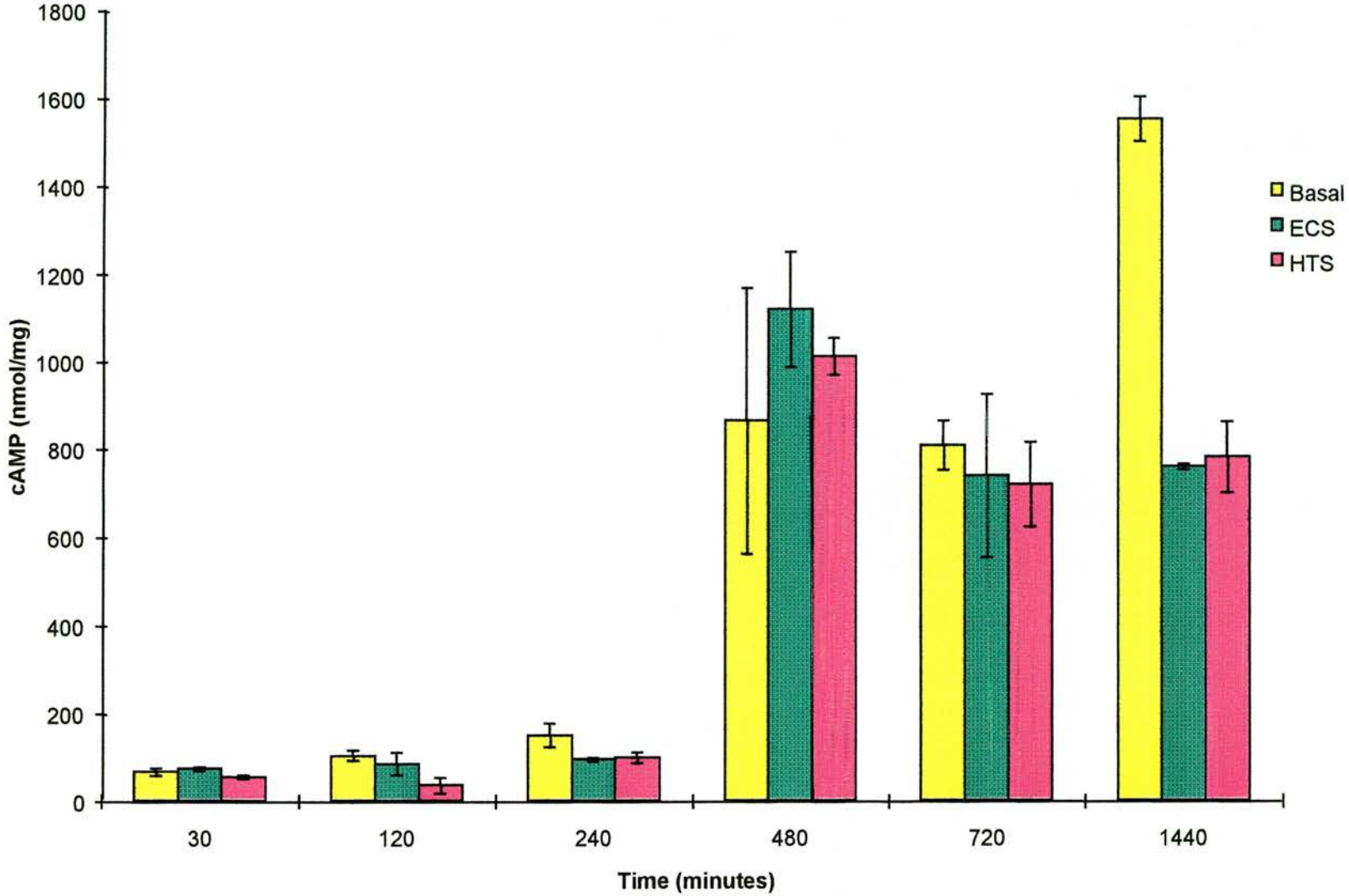


Figure 5.09: cAMP accumulation in a JPO9 cells following stimulation with pooled sera from euthyroid and hyperthyroid cats and TRABs positive human Grave's patients at 1, 2 and 5% v/v final concentration over 1440 minutes (24 hours) in the presence of 800uM IBMX. Results shown are those of the mean of triplicate wells ± SEM. ECS, euthyroid cat serum; HTS, hyperthyroid cat serum.

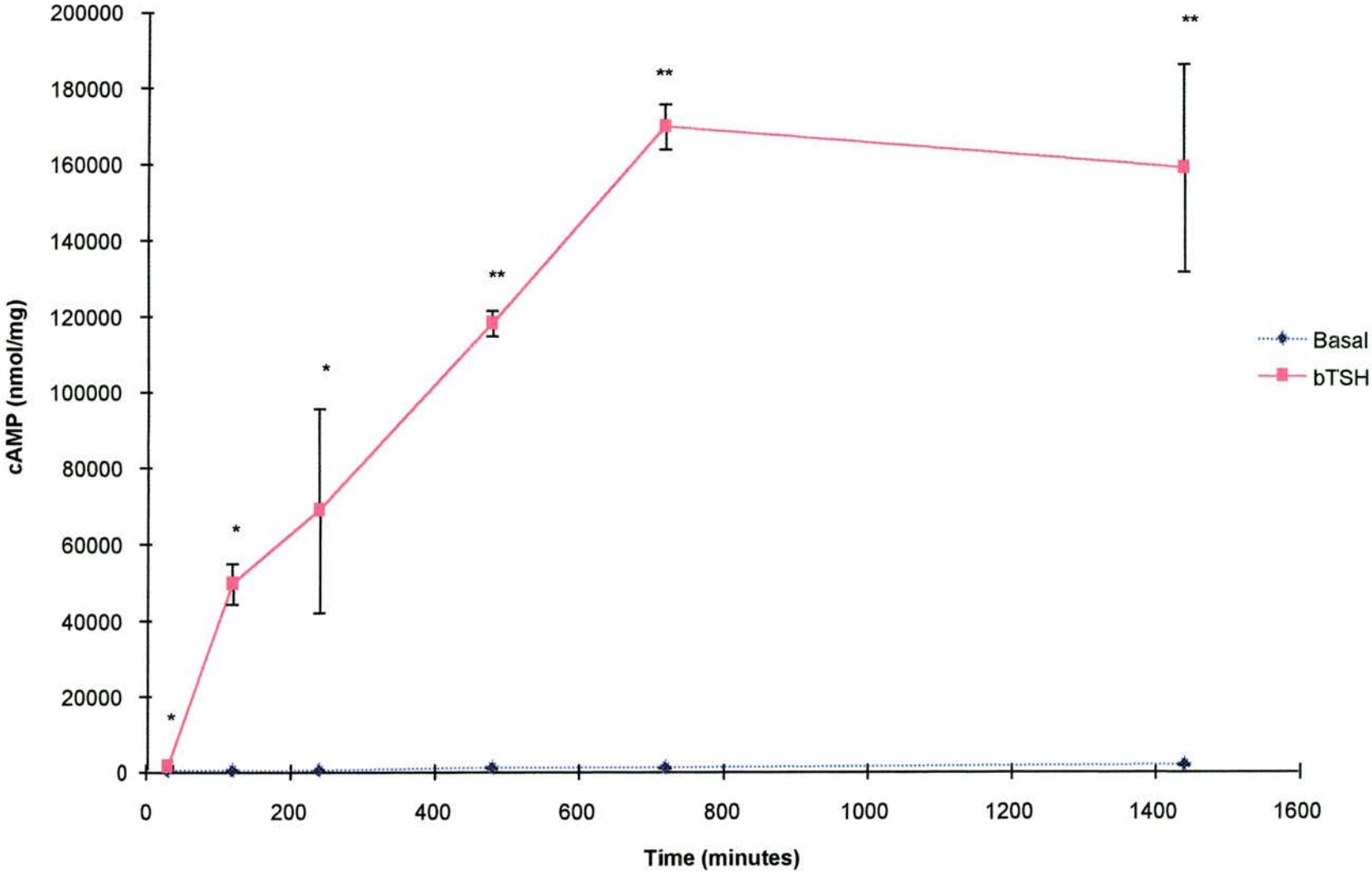


Figure 5.10: cAMP accumulation in JPO9 cells following stimulation with bTSH (500mU/l) over 24 hours in the presence of 800uM IBMX. Results shown are those of the mean of triplicate wells ± SEM. $p<0.05$*,$p<0.01$** from basal. bTSH, bovine TSH.

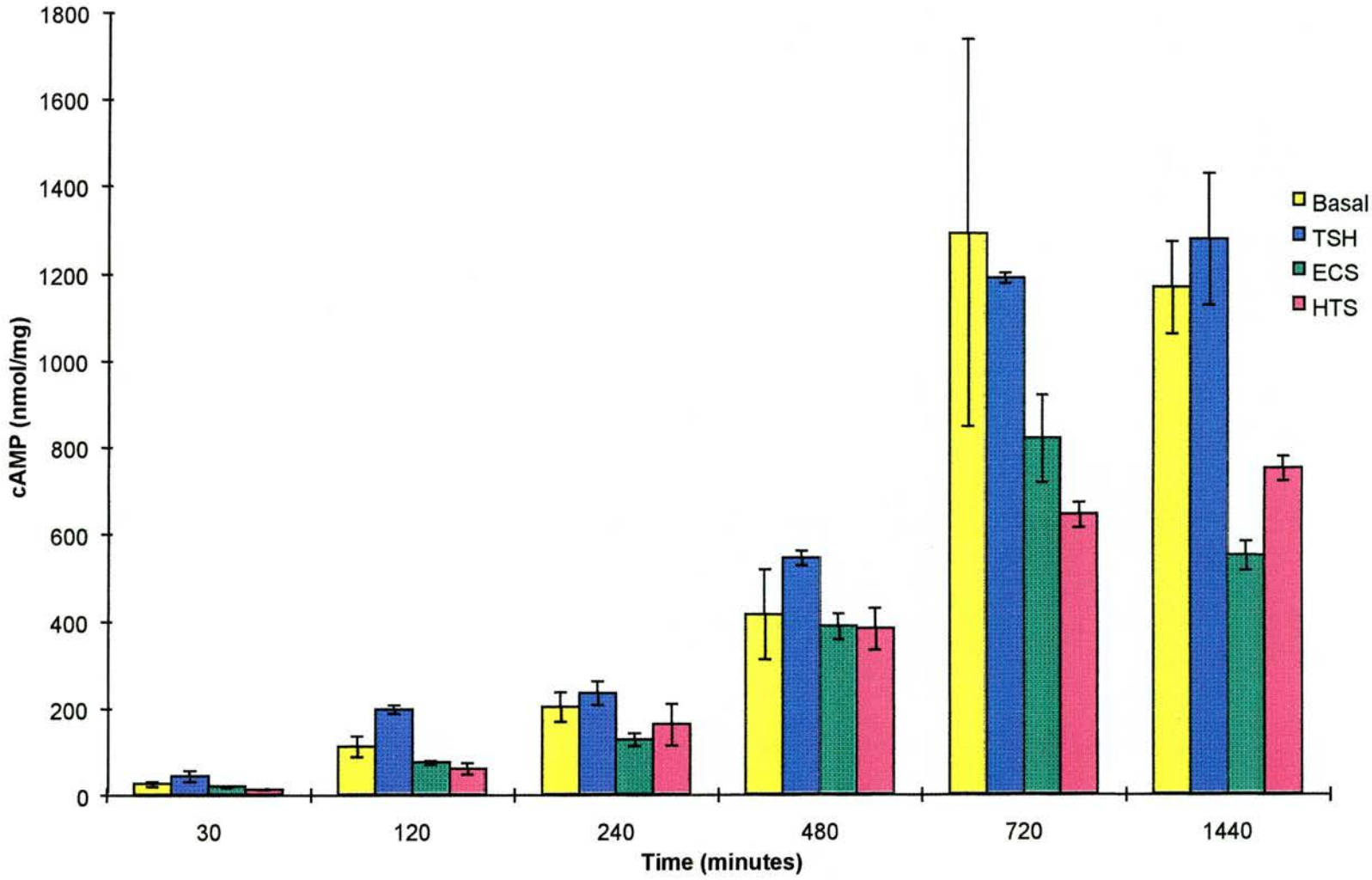


Figure 5.11: cAMP accumulation in JPO2 cells following stimulation with bTSH (500mU/l), pooled sera from euthyroid and hyperthyroid cats and TRABs positive human Graves' disease patients at 1, 2 and 5% v/v final concentration over 1440 minutes (24 hours) in the presence of 800uM IBMX. Results shown are those of the mean of triplicate wells ± SEM. ECS, euthyroid cat serum; HTS, hyperthyroid cat serum

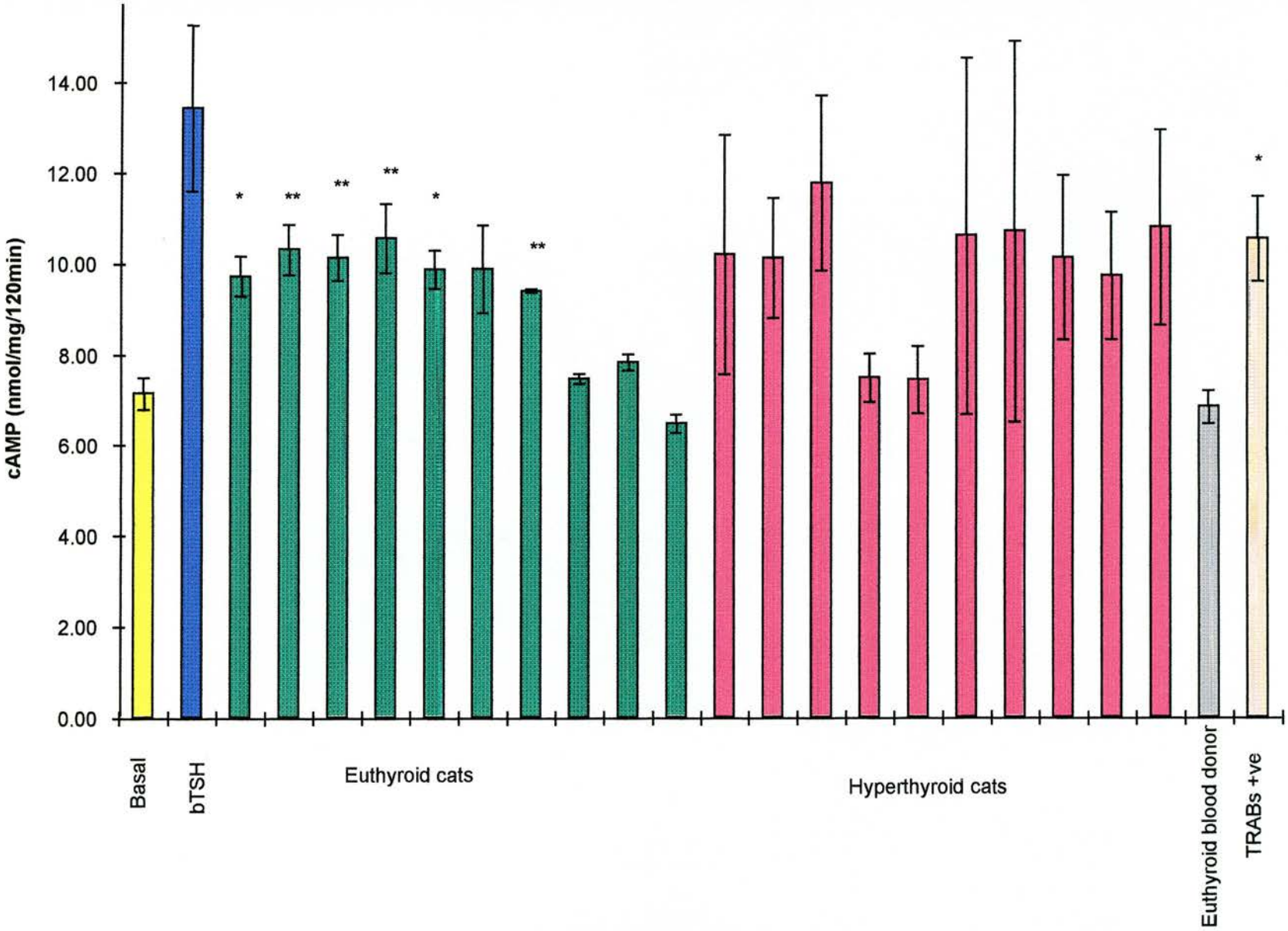


Figure 5.12: cAMP accumulation in JPO9 Cells following stimulation with 5% serum final concentration from euthyroid and hyperthyroid cats and TRABs positive and euthyroid blood donor over 120 minutes in the presence of 800uM IBMX. Results shown are those of the mean of triplicate wells ± SEM. $p<0.05$*,$p<0.01$** from basal. TRABs, TSH receptor antibody; bTSH, bovine TSH.

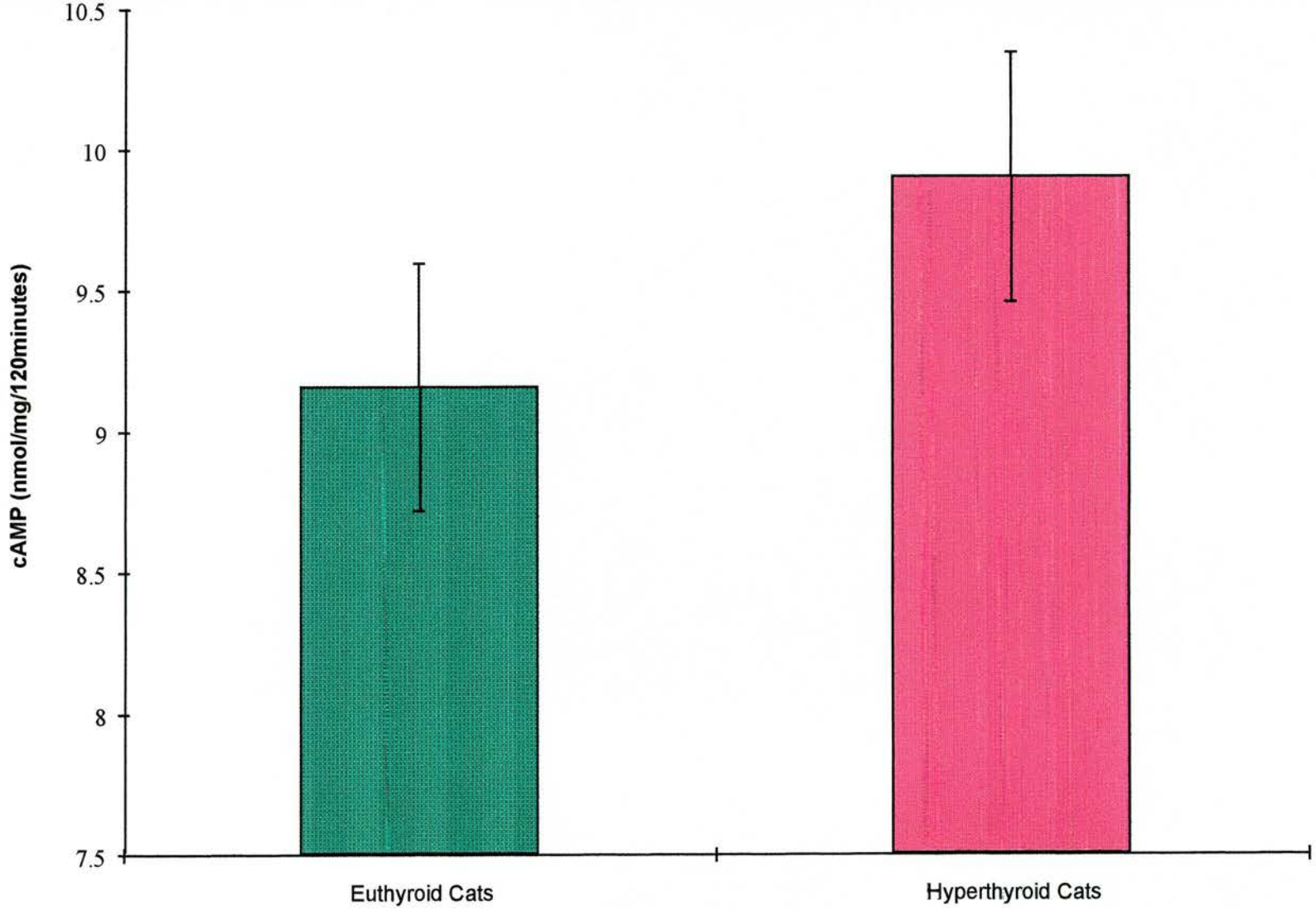


Figure 5.13: cAMP accumulation in JPO9 Cells following stimulation with 5% serum final concentration over 120 minutes in the presence of 800uM IBMX. Comparison between euthyroid and hyperthyroid cats (n=10 for each group). Results shown are those of the mean of triplicate wells ± SEM.

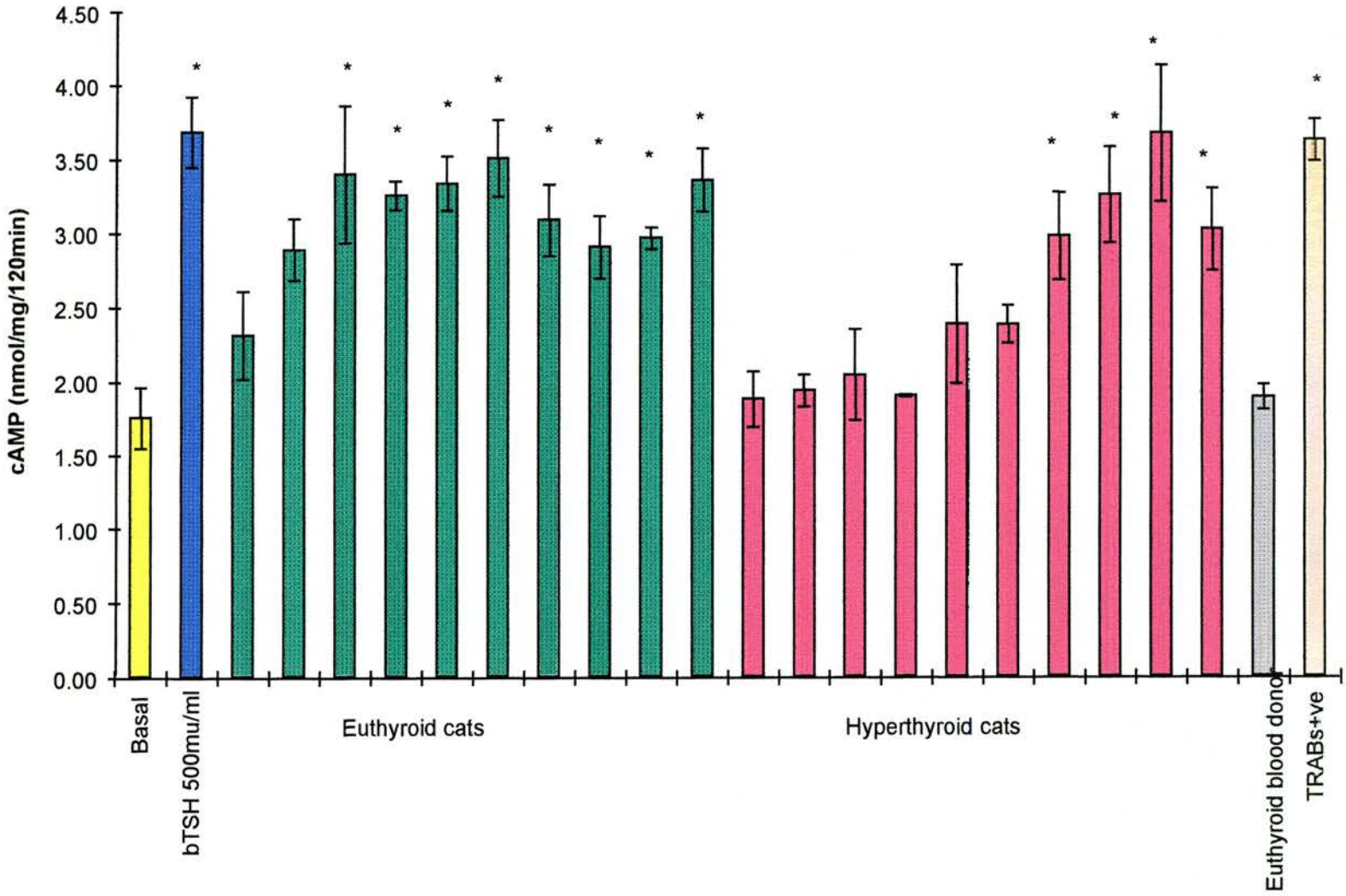


Figure 5.14: cAMP accumulation in JPO9 Cells following stimulation 1mg/ml final concentration purified IgG over 120 minutes in the presence of 800uM IBMX. Results shown are those of the mean of triplicate wells ± SEM. $p<0.05$* from basal. bTSH, bovine TSH; TRABs, TSH receptor antibody.

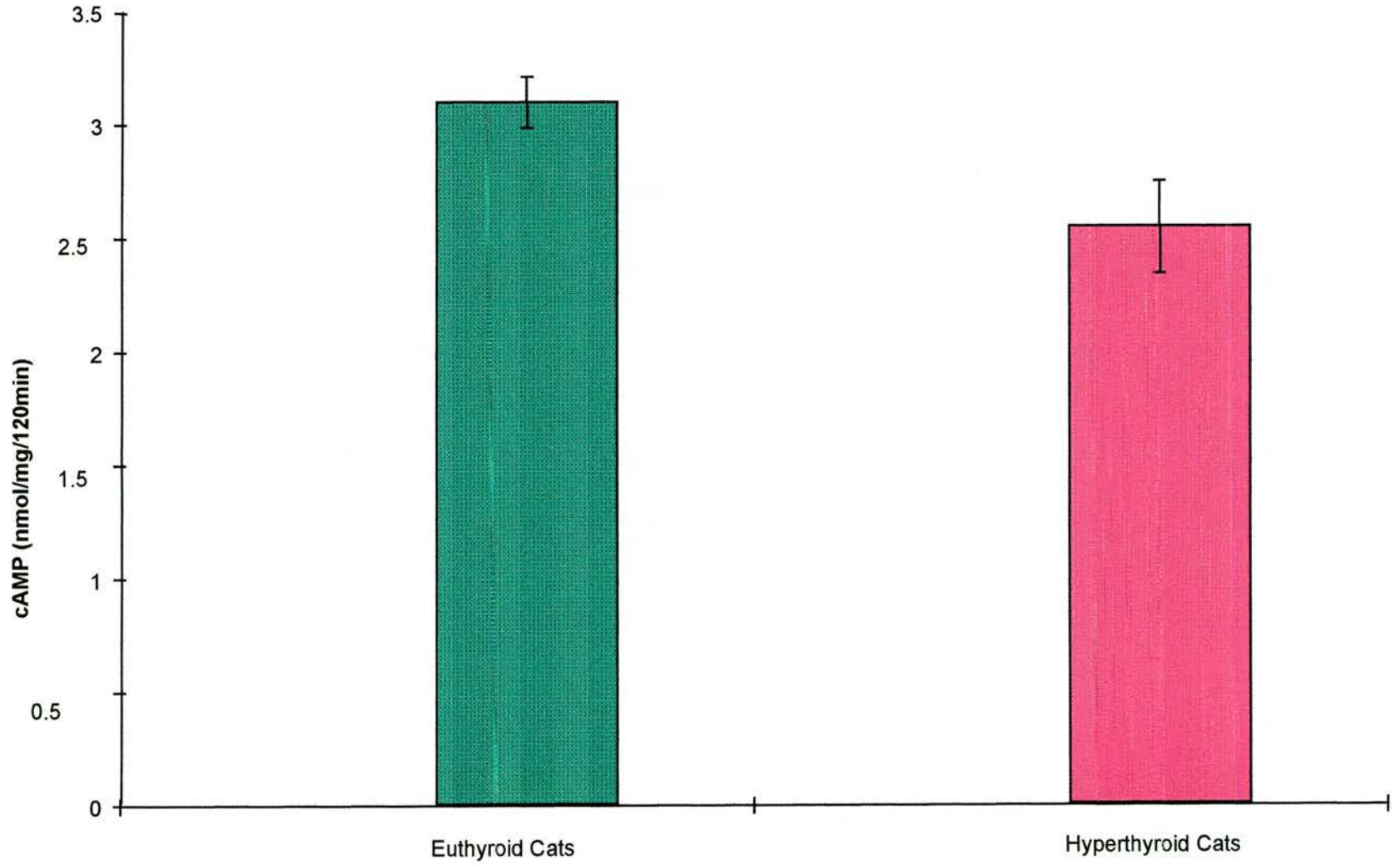


Figure 5.15: cAMP accumulation in JPO9 Cells following stimulation 1mg/ml purified IgG final concentration over 120 minutes in the presence of 800uM IBMX. Comparison between euthyroid and hyperthyroid cats (n=10 for each group). Results shown are those of the mean of triplicate wells ± SEM. $p<0.05$*.

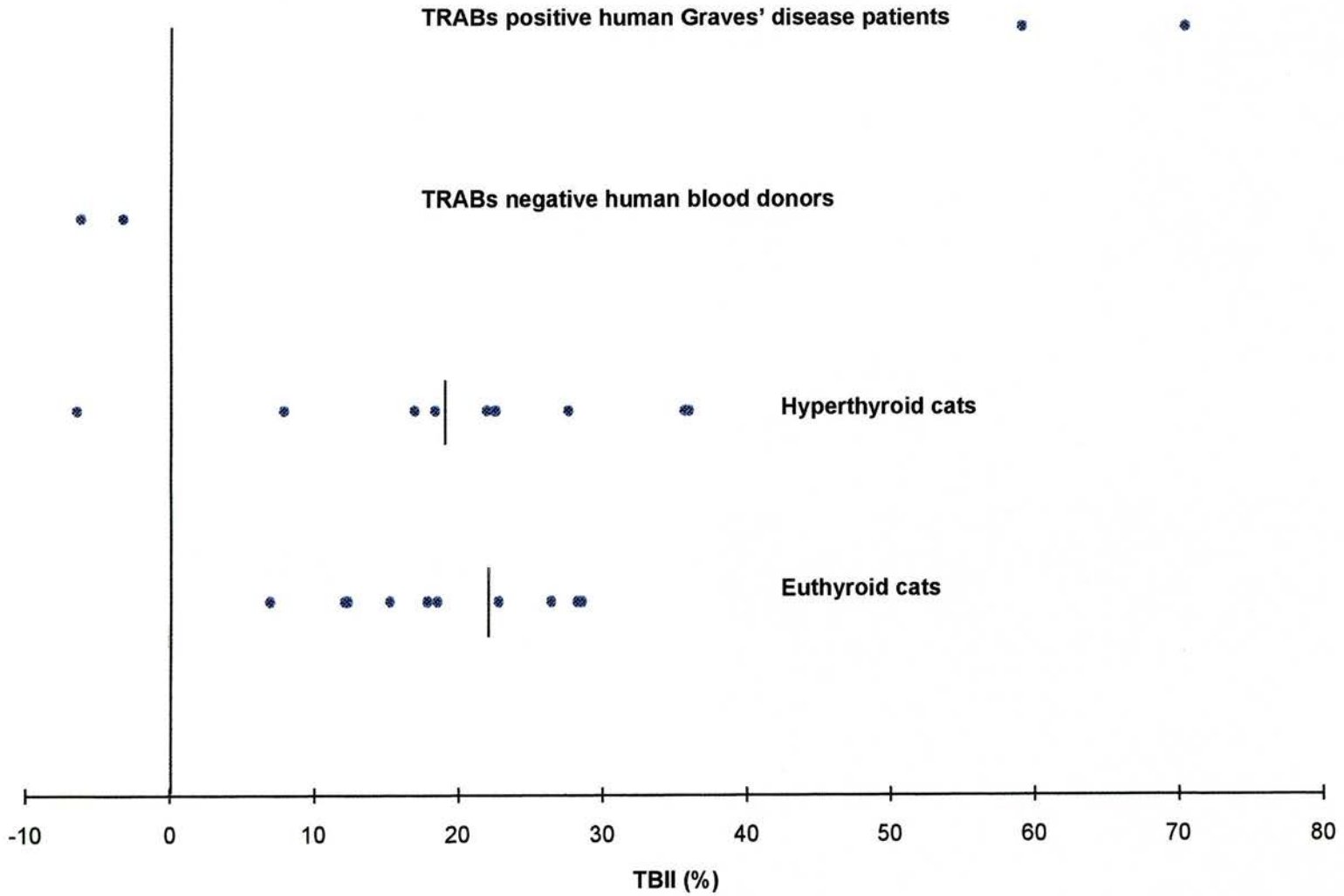


Figure 5.16: Thyrotropin receptor antibodies in sera of euthyroid (n = 10) and hyperthyroid cats (n = 10), and euthyroid (n = 2) and TRABs positive (n = 2) humans . Results shown are percentage of inhibition of binding of TSH to porcine thyrocyte membranes (TBII). Solid line represents the median.

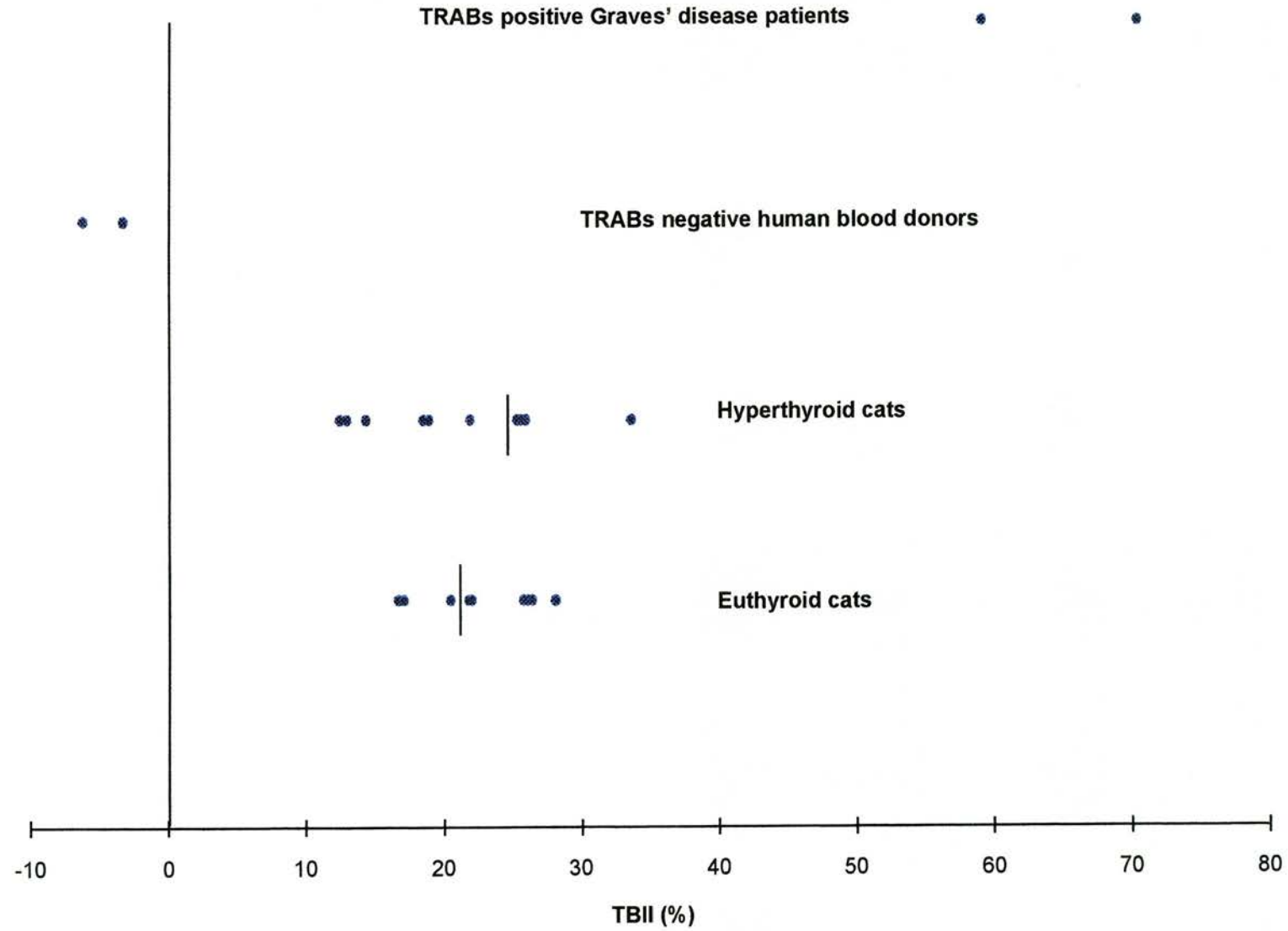


Figure 5.17: Thyrotropin receptor antibodies in samples of purified IgG from euthyroid and hyperthyroid cats. Results shown are percentage of inhibition of binding of TSH to porcine thyrocyte membranes (TBII). Solid line represents the median.

5.04 DISCUSSION

Feline thyrocytes demonstrate cAMP and phosphoinositol responses to bTSH. In the present study, the cAMP response in feline thyrocytes to bTSH was approximately 25 to 500 fold less than the response in JPO9 cells. This reflects either the increased number of TSH receptors expressed in JPO9 cells (Harfst, Ross, Nussey *et al.,* 1994), or differences in the sensitivity of the TSH receptors to bTSH between species. The stimulation of the phosphoinositol cascade in feline thyrocytes was similar to that observed in JPO9 cells. Feline thyrocytes isolated in the manner described showed a cAMP response to TSH and to sera from TRABs positive patients demonstrating that they are a suitable bioassay system for the investigation of TSIs in feline hyperthyroidism. The development of such a bioassay system is important to help reduce the problems of sensitivity and specificity that cross-species studies may produce.

While each of the cell types investigated responded to bTSH and human serum containing TRABs, this study was unable to detect the presence of any significant TSI, or TBII-like activity in the sera of hyperthyroid cats. This was the case when using a number of cell types and systems to assay for such antibodies. Thus it was not possible to detect the presence of any immunoglobulins or humoral agents in serum that could be responsible for the pathogenesis of feline nodular goitre.

A number of assay systems for cAMP production gave positive results for both euthyroid and hyperthyroid cat serum and IgG preparations, illustrating the lack of specificity of such systems for investigating specific interactions with the TSH receptor. Cells have a number of receptors that may lead to changes in cAMP production. The expression of these receptors and their activation (including that of the TSH receptor) may vary depending on the environment of the cell. The lack of any significant differences between euthyroid and hyperthyroid cats in cAMP accumulation in the culture medium from serum (whether pooled or from individual cats) in primary cat cell preparations (FNG and FT), FRTL-5 and JPO9 cells is a consistent finding in these experiments. Additionally, purified immunoglobulins from hyperthyroid cats also showed no significant cAMP accumulation compared to euthyroid cats. This is in agreement with the study of Brown, Keating, Livingstone *et al.* (1992), who also found no significant cAMP stimulation with purified IgG from hyperthyroid cats using FRTL-5 cells despite demonstrating a positive growth effect in such cells. It is possible that growth in FRTL-5 cells may be due to the activation of

alternative pathways to cAMP. Alternatively, it may be related to artefacts caused by a problem of species-specificity (i.e. using a rat thyrocyte cell line to demonstrate feline TSI activity) .

In addition to being unable to demonstrate cAMP stimulating activity in hyperthyroid cat serum above that seen in euthyroid cat serum, it was not possible in these experiments to demonstrate a difference in the TBII in sera or purified IgG preparations of hyperthyroid cats when compared to euthyroid cats. This is in contrast to the study by Brown, Keating, Livingstone *et al.* (1992), who, with a similar assay system, reported that with purified IgG preparations, three of nine (33%) hyperthyroid cats exhibited characteristics that suggested the presence of TBIIs. The mean TBII of hyperthyroid cats (6.5%) was also significantly greater than the mean TBII of euthyroid cats (1.1%)($p < 0.01$). Brown's group also demonstrated that when purified IgG was used and porcine thyrocyte membranes were substituted for cat thyrocyte membranes, eight out of 10 (80 per cent) hyperthyroid cats were positive (TBII of greater than 50%). The mean TBII index of hyperthyroid cats (82.5%) was significantly greater than euthyroid cats (0.3%)($p < 0.001$). The large difference between the mean TBII of hyperthyroid cats when porcine thyrocyte membranes where used in place of feline thyrocyte membranes, may be explained by the fact that the same IgG preparations were not used in each experiment. Furthermore, different buffers were also used between these assays (Tris buffer for porcine membranes, and low-NaCl Tris buffer for feline membranes) which was an additional confounding factor in these experiments. Brown, Jackson, Pohl *et al.* (1978), have previously demonstrated that low-NaCl Tris buffer increases the sensitivity of this assay system, which could easily explain the difference in TBII index between her group's two experiments. In the present study, although six of 10 euthyroid cats had TBIIs of greater than 0%, none were greater than 50%.

In our hands, purified immunoglobulins at the same concentrations as Brown, Keating, Livingstone *et al.* (1992) (1mg/ml) in low-NaCl buffer, could not be used undiluted in this assay as they caused significant non-specific displacement of TSH binding regardless of whether they were from euthyroid or hyperthyroid cats. Samples, therefore, had to be diluted with TRABs negative serum to achieve meaningful results. There are two possible sequelae to this, one being that Brown, Keating, Livingstone *et al.* (1992) were reporting TBII activity that was in fact non-specific displacement and by chance happened to find an effect in thyrotoxic cats more commonly than euthyroid cats, or, alternatively, it is possible that the dilution with serum used in the present experiments resulted in the dilution of TBII activity in our assay system, or more likely, changes in matrix effects altering the binding of receptor antibodies.

While the presence of thyroid stimulating immunoglobulins is well-defined as the major factor in the pathophysiology of Graves' disease in humans, there is still controversy over the existence and significance of thyroid stimulating immunoglobulins in non-immunogenic forms of thyrotoxicosis in humans. The variability of assay systems to detect immunoglobulins which interact with the TSH receptor has led to lead to the majority of debate. It is a common finding that various forms of thyrotoxicosis other than Graves' disease have variable presence of TSIs, TBIIs and TGIs, and are not necessarily related to clinical parameters of thyrotoxicosis such as age of patient, duration of disease or severity of thyroid disease (Goretzki, West, Koob *et al.,* 1987; Brown, 1995). Euthyroid humans with non-thyroidal illness may also demonstrate positive TRABs titres although it is unclear if these are caused by the presence of similar IgGs which bind to the TSH receptor, or other substances (Zakarija and McKenzie, 1987; Beckett and Toft, personal communication, 1998).

The problems with such *in vitro* tests is that factors other than IgG may be present in the serum matrix that can lead to responses in crude bioassay systems which are, in effect, assay artefact. It is thus essential that comparative responses are studied between affected and unaffected individuals. A number of studies reporting the presence of TSIs and TBIIs in thyroid disease other than Graves' disease do not compare these groups with healthy control subjects (Goretzki, West, Koob *et al.,* 1987). Using such a comparison, the present studies have been unable to demonstrate any such differences between euthyroid and thyrotoxic cats.

Although histological changes in the thyroid glands of cats with thyrotoxicosis and in humans with acute toxic nodular goitre (ATN) (Grubeck-Lobenstein, Derfler, Kassal *et al.*, 1985) compatible with Graves' disease have been reported, little convincing evidence as to the presence of TSIs in the pathogenesis of feline hyperthyroidism has been published. Peterson, Livingstone and Brown, (1987), could find no evidence of TSIs in 23 hyperthyroid cats using FRTL-5 cells and cAMP as reporters. Thyroid microsomal and nuclear autoantibodies have been demonstrated in 10 of 29 (34 per cent) of thyrotoxic cats (Kennedy and Thoday, 1988) compared to none of the control cats. However, these autoantibodies appeared non-functional (and, perhaps, therefore non-specific) as Kennedy, Thoday and Mooney (1989), could find no evidence of TSI activity in 29 hyperthyroid cats using iodine uptake in FRTL-5 cells as a marker. In contrast, sera from Graves' disease patients in the same assay system significantly increased iodine uptake.

There are a number of reasons to suggest that it would be unusual to detect positive TSIs and TBIIs in the serum of hyperthyroid cats. The first is that nodular thyroid tissue from cats with hyperthyroidism transplanted into nude mice continues to grow, remains hyperfunctional and their growth is not stimulated by injecting sera from thyrotoxic cats into these mice (Peter, Gerber, Studer *et al.,* 1987). This study provided convincing evidence that even if TSIs are involved in the pathogenesis of feline hyperthyroidism, they are not necessary for the continuation of the disease. Indeed, without the presence of other markers of autoimmunity such as heredity and histological evidence of autoimmunity such as lymphocytic infiltration, the presence of TSIs, TBIIs or TGIs, the significance of these putative immunoglobulins in the pathogenesis of the disease is questionable.

It is possible that the lack of evidence for TSIs or TBIIs in this study may reflect the UK cat population that has been studied. It would appear that in the USA, the cats being presented for diagnosis and treatment of thyrotoxicosis have a less severe clinical presentation than those cats in which the disease was first reported (Broussard, Peterson and Fox, 1995) It is possible, therefore, that cats with thyrotoxicosis in the USA (where Brown's feline samples were obtained) are being diagnosed at an earlier stage of the disease and thus may have the humoral agent still present. In humans it is believed that TBIIs and TGIs appear early in the pathogenesis of some forms of goitre and then disappear. Our data cannot exclude such a possibility occurring in cats. A large prospective study in cats following their thyroid hormone status, thyroid disease progression and TGI/TSI/TBII potential over time may help to determine this, but due to ethical considerations, would be difficult to pursue. Furthermore, iodine intake of the cats in Brown's study may have been radically different to the cats in our study. Iodine deficiency is known to induce thyroid growth and human patients with endemic goitre have been found to have TGIs in one study (van der Gaag, Drexhage, Wiersinga *et al.,* 1985) but not another (Vitti, Chiovato, Tonacchere *et al.,* 1994). Iodine deficiency is known to directly enhance thyroid sensitivity to growth stimuli even in normal individuals, although this is relatively uncommon. This effect is enhanced further in certain cases of thyroid disease such as Hashimoto's thyroiditis, treated euthyroid Graves' disease patients, subclinical hypothyroidism, transient post-partum thyroiditis, hemithyroidectomy for benign nodules and euthyroid patients following previous amiodarone induced destructive thyrotoxicosis (Roti and Vagenakis, 1996).

The differences between our study and that of Brown, Keating, Livingstone *et al.*, (1992) may also be explained by laboratory methods. As previously discussed, many human studies using

similar methods have published conflicting reports on the existence of TSIs. Whatever the consequence, the fact that both human (Smeds, Peter, Gerber *et al.,* 1988) and feline (Peter, Gerber, Studer *et al.,* 1987) adenomas are truly autonomous and do not require the presence of a humoral factor for growth or stimulation of function, would confirm that these nodules are TSI/TBII independent. Indeed, it is now well known that the primary pathological event in the generation of adenomas in human nodular goitre is somatic mutations in the TSH receptor or its associated G protein (see Chapters 1.0 and 6.0).

6.00 MUTATIONAL ANALYSIS OF THE FELINE THYROTROPIN RECEPTOR

6.01 INTRODUCTION

Somatic mutations of the TSHR and its associated G-proteins are now recognised as the major cause of toxic nodular goitre (functional adenomas) in humans. Germline TSHR gene mutations also result in the autosomal dominant syndrome of hereditary toxic thyroid hyperplasia, which is characterised by biochemical and clinical evidence of thyrotoxicosis from birth. In both of these syndromes, the TSHR mutations occur within exon 10 of the gene (codons 450 to 680), which encodes the seven transmembrane regions, and the extracellular and intracellular loop of the receptor.

The pathogenesis of feline hyperthyroidism is unclear but is speculated to be mutifactorial. Previous investigations into the pathogenesis of feline thyrotoxicosis are discussed in Chapter 1.03 and 1.04.

The aim of this study was to

- examine the gene for the feline TSHR in the region of codon 480 to 640 to determine if gain of function somatic mutations are a common cause of toxic nodular goitre in cats.

6.02 MATERIALS AND METHODS

a) PATIENT DATA

The clinical data of 11 cats with sporadic thyrotoxicosis and two littermates (who had the same owner) who were also thyrotoxic, are represented in Table 6.01. No history of thyroid disease in the mother, 2 other siblings, or 3 half-siblings of the related cats could be ascertained from their owners. Thyroid tissues from cats I to M was not evaluated histologically but macroscopically they appeared enlarged, hyperaemic, and contained multiple nodular areas.

b) METHODS

Genomic DNA was obtained from the thyroid tissue from glands removed for the treatment of the disease, peripheral blood leucocytes and two normal cat ovariohysterectomy samples. Single stranded conformational polymorphisms (SSCPs) were constructed and the DNA sequences of SSCPs of the PCR products were determined by semiautomated cycle sequencing. The normal sequence of the feline TSHR was determined from ovariohysterectomy samples using "Sequence Navigator" software. Methodological details are described in detail in Chapter 2.046. The molecular biology was performed by Dr Simon Pearce of the Endocrine Unit, Department of Medicine, University of Newcastle upon Tyne, U.K.

6.03 RESULTS

a) NORMAL FELINE TSHR SEQUENCE

The previously uncharacterised normal feline TSHR DNA sequence between codons 480 to 640 was determined (Figure 6.01). The amino acid sequence over this transmembrane region was found to be highly homologous to that of the cloned mammalian TSHRs with 95%, 92% and 90% amino acid identity between the derived feline TSHR sequence and canine, human and bovine TSHRs respectively (Figure 6.02).

b) MUTATIONAL ANALYSIS

The DNA screening method of SSCP analysis was performed on thyroid DNA from the 11 cats with sporadic thyrotoxicosis, on leucocyte DNA from the two thyrotoxic littermates, and two samples from healthy cats as controls. No abnormal SSCPs were detected in DNA samples from any of the 13 thyrotoxic animals. To confirm the findings of the SSCP analysis, direct DNA sequence analysis of codons 480 to 640 of the feline TSHR was performed on thyroid DNA from seven of the thyrotoxic cats (cats A, B, C, F, I J and L). This analysis confirmed the normal cat TSHR sequence and failed to show any DNA sequence abnormality.

Table 6.01: Clinical data from the 13 cats with thyrotoxicosis screened for thyrotropin receptor somatic mutations

Cat	Age	Sex	Breed	Serum T4 conc. (nmol/l)	Medical treatment	Surgical treatment	Lobe involvement	Histopathology
A	13	MN	DSH	147	PP+KI	Yes	Unilateral	Adenomatous Hyperplasia
B	15	FN	DLH	176	PP+KI	Yes	Bilateral	Adenomatous Hyperplasia
C	11	FN	DSH	163	CBZ	Yes	Bilateral	Adenomatous Hyperplasia
D	12	FN	DSH	103	CBZ	Yes	Bilateral	Adenomatous Hyperplasia
E	10	MN	DSH	60	NO	No	Bilateral	Adenomatous Hyperplasia
F	12	MN	DSH	173	CBZ	Yes	Bilateral	Adenomatous Hyperplasia
G	20	FN	DLH	50	NO	No	Bilateral	Adenomatous Hyperplasia
H	13	FN	DSH	76	CBZ	No	Bilateral	Adenomatous Hyperplasia
I	8	MN	DSH	194	CBZ	Yes	Bilateral	Unknown
J	12	MN	DSH	100	CBZ	Yes	Unilateral	Unknown
K	13	FN	DSH	94	CBZ	Yes	Unilateral	Unknown
L	11	FN	DSH	107	CBZ	Yes	Unilateral	Unknown
M	17	FN	DSH	121	CBZ	Yes	Unilateral	Unknown

CBZ, carbimazole; DLH, Domestic longhair; DSH, Domestic shorthair; FN, ovariohysterectomised female; KI, potassium iodate; MN, castrated male; PP, propranolol; T4, thyroxine.

```
Codon  GAG TAC TAC AAC CAT GCC ATT GAA TGG CAG ACA GGC CCT GGA TGC AAT GCG GCT GGT TTC TTC ACT
480    E   Y   Y   N   H   A   I   E   W   Q   T   G   P   G   C   N   A   A   G   F   F   T

       GTC TTT GCC AGT GAA TTG TCA GTG TAT ACA CTG ACA GTC ATC ACT CTG GAG CGC TGG TAT GCC ATT
502    V   F   A   S   E   L   S   V   Y   T   L   T   V   I   T   L   E   R   W   Y   A   I

       ACC TTC GCC ATG CGC CTG GAC CGG AAG ATG CGC CTC AGG CAC GCA TAT GCC ATC ATG GTT GGG GGC
524    T   F   A   M   R   L   D   R   K   M   R   L   R   H   A   Y   A   I   M   V   G   G

       TGG GTT TGC TGC TTC CTG CTG GCC CTG CTC CCT TTG GTG GGA ATA AGC AGC TAT GCC AAG GTC AGC
546    W   V   C   C   F   L   L   A   L   L   P   L   V   G   I   S   S   Y   A   K   V   S

       ATC TGC CTG CCC ATG GAC ACT GAA ACA CCT CTT GCC CTG GCA TAT ATT ATC CTT GTC CTG TTG CTC
568    I   C   L   P   M   D   T   E   T   P   L   A   L   A   Y   I   I   L   V   L   L   L

       AAC ATA GTC GCC TTT ATC ATT GTC TGC TCC TGT TAC GTG AAA ATC TAC ATC ACA GTC CGG AAT CCC
590    N   I   V   A   F   I   I   V   C   S   C   Y   V   K   I   Y   I   T   V   R   N   P

       CAG TAC AAC ACC GGG GAC AAA GAC ACC AAA ATT GCG GAA AGG ATG GCT GTG TTA ATC TTC ACT GAC
612    Q   Y   N   T   G   D   K   D   T   K   I   A   E   R   M   A   V   L   I   F   T   D

       TTC ATT TGC ATG GCT CCA ATC TCA
634    F   I   C   M   A   P   I   S
```

Figure 6.01: DNA and amino acid sequence of the feline thyrotropin receptor gene. The DNA sequence of the feline TSHR is shown from codon 480 to 640 on the top line. The amino acid translation is shown below, with the codon numbering corresponding to that found in the homologous region of the human TSHR. The DNA sequence was derived from analysis of normal feline uterine tissue. Amino acids are shown in conventional single letter code.

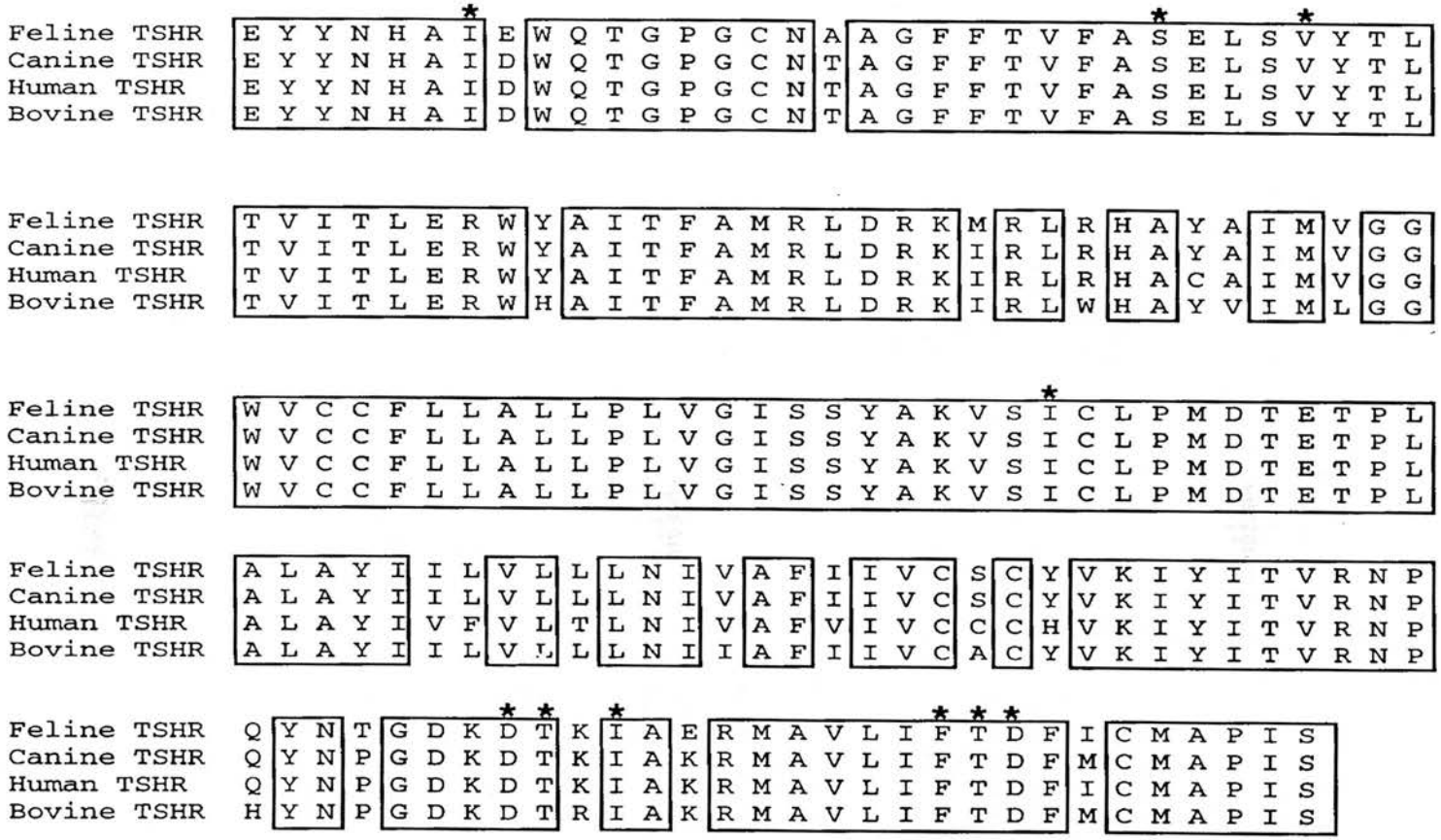


Figure 6.02: Alignment of codons 480 to 640 of the feline, canine, human and bovine thyrotropin receptors. The amino acid sequences of the canine, human and bovine TSHRs were aligned with that derived for the feline TSHR using the 'pileup' program of the Genetics Computing Group, University of Wisconsin. Areas of amino acid identity between all four receptors are shown as boxed. The sites of known activating mutations of the human TSHR found in toxic follicular adenoma or hereditary toxic thyroid hyperplasia are marked with an asterix.

6.04 DISCUSSION

Mutations of the TSHR can result in loss or gain of function. Somatic 'gain of function' mutations are the major cause of benign toxic thyroid adenomas and of some cases of multinodular goitre in humans (Duprez, Parma, Van Sande *et al.*, 1998). For this reason, we decided to investigate the TSHR of feline thyroid adenomas to determine if somatic mutations of the TSHR are involved in the pathogenesis of feline thyrotoxicosis

Hyperfunctioning thyroid adenomas are responsible for 5 to 30 per cent of cases of human thyrotoxicosis (Ledent, Parma, Dumont *et al.*, 1994). The majority of somatic mutations occur in the tenth exon of the TSHR which encodes for the domain of the active G protein (G_s). The mutation is confined to the adenomatous tissue demonstrating the monoclonal nature of solitary adenomas (Ledent, Parma, Dumont *et al.*, 1994). In human toxic nodular goitre, gain of function mutations are scattered over most of the carboxyl half of the TSHR in the first and second extratracellular loops, the third, sixth and seventh transmembrane segments and the third intracellular loop, and more recently described, deletions in the third intracellular loop (Wonerow, Schoneberg, Schultz *et al.*, 1998). These mutations have been shown to be functional by transfection into COS cells and demonstrating increased basal cAMP accumulation over wild type. In the only series where exon 10 of the TSHR was entirely sequenced, 81 per cent of toxic adenomas harboured an activating mutation (Parma, Van Sande, Swillens *et al.*, 1995), making somatic mutations of the TSHR the most common cause of toxic nodular goitre in humans. Mutations of the stimulating G-protein ($G_{s\alpha}$) probably account for the remainder of cases (Ledent, Parma, Dumont *et al.*, 1994). There are known 'hot spots' for somatic functional TSHR mutations in humans. These include Ser281, Ile486, Ile586, Phe631, and Asp 633 (Parma, Duprez, Van Sande *et al.*, 1997). Whilst there is emerging agreement on the prevalence of TSHR and $G_{s\alpha}$ mutations in human patients with toxic nodular goitre, there are still some 12 per cent of cases that do not have demonstrable mutations, leaving room for other pathophysiological mechanisms or gene targets (Duprez, Parma, Van Sande *et al.*, 1998).

Our DNA sequencing and SSCP studies have not demonstrated TSHR gene abnormalities between codons 480 and 640 in thyroid DNA from 11 cases of sporadic feline thyrotoxicosis and in leukocyte DNA from 2 littermates with familial feline thyrotoxicosis. In human toxic follicular

thyroid adenomas and hereditary toxic thyroid hyperplasia, 13 of the 17 reported TSHR gene mutations have occurred between codons 480 and 640. If mutations of the feline TSHR are able to cause a gain of function, such mutations are likely to occur in the same area, because of the 92% amino acid retention of identity between the human and feline TSHRs that we have demonstrated in this region. However, our study did not examine the whole of the feline TSHR coding sequence for such mutations, and we cannot exclude the possibility that the molecular dynamics of feline TSHR activation are significantly different from those of the human TSHR, such that gain of function mutations of the feline TSHR occur in regions of the gene we have not examined.

Single strand conformational polymorphism (SSCP) analysis is the most widely used method to demonstrate somatic mutations. It is simple and versatile and works on the principle that single-stranded DNA molecules adopt specific secondary structures under denaturing conditions (Tonacchera, Cetani, Van Sande *et al.*, 1996). This method is useful as a screening test, but can lead to false negative results if not used with another method, such as DNA sequencing (Tonacchera, Cetani, Van Sande *et al.*, 1996). We have used both of these techniques and have found no mutations in the DNA from the TSHR in feline adenomas.

The molecular genetic approach that we have taken in this study may be more widely applicable to the investigation of feline thyrotoxicosis, as a variety of genetic abnormalities have been demonstrated in differerent forms of human thyrotoxicosis, such as point mutations of the stimulatory G-protein α-subunit in toxic follicular adenomas or monoclonality of nodules within multinodular goitres. Our analysis suggests that feline thyrotoxicosis is not commonly caused by TSHR gene mutations and does not therefore provide an animal model for the study of somatic or hereditary human toxic follicular adenomas. Further investigation into feline thyrotoxicosis may yield insights into the pathogenesis of human thyrotoxicosis and provide a much needed spontaneous model for at least one form of the human disorder.

7.00 THYROIDAL SELENOENZYME EXPRESSION AND SELENIUM STATUS IN THE DOMESTIC CAT

7.01 INTRODUCTION

The thyroid gland contains more selenium per gram weight than any other tissue suggesting an important role for this trace element in the normal homeostasis of the gland. Selenium acts through the expression of selenoproteins and in the thyroid gland these include glutathione peroxidase (GPX) which catalyses the production of H_2O_2 and protects the thyrocyte from oxidative damage and thioredoxin reductase (TR), which also has oxidant protective properties and has been shown to be involved in the regulation of growth of normal and tumourous cells. Deiodination is also catalysed in the thyroid gland by the iodothyronine deiodinases but this family of selenoenzymes are not expressed in the thyroid tissue of all species.

Selenium status has been reported to influence thyroid hormone status and metabolism in rats and humans. Conversely, thyrotoxicosis (resulting from Graves' disease and autonomous nodules) results in lowering of serum selenium concentrations and whole blood GPX activity (Beckett, Peterson, Choudhury *et al.*, 1991).

The expression of human thyroidal TR has been shown to be increased by PMA and the calcium ionophore A23187 (Beckett, Howie, Nicol *et* al., 1998; Howie, Arthur, Nicol *et al.,* 1998). TR also appears to have an important role in cell proliferation. TR has been shown to act as an intracellular growth factor for both normal and tumour cells in culture (Berggren, Gallegos, Gasdaska *et al.*, 1996; Gallegos, Gasdaska, Taylor, 1996) by activating transcription factors that lead to cell division (Powis, Gasdaska, Gasdaska *et al.,* 1997). To date, no TR binding sites have been found on the surface of cancer cells. TR also acts in an autocrine manner by enhancing the cellular response to other growth factors (Berggren, Gallegos, Gasdaska *et al.*, 1996; Gasdaska, Kirkpatrick, Monfort *et al.,* 1996; Powis, Gasdaska, Gasdaska *et al.,* 1997).

Selenium status can also modify TR expression/activity. TR activity is inhibited by high concentrations of selenite (Bjornstedt, Kumar and Holgrem, 1995) and, *in vivo*, TR activity in rat liver, lung and kidney undergoes transitory increased expression when these animals are fed a high selenium diet (Berggren, Mangin, Gasdaska *et al.*, 1999).

Selenium- and iodine-status are closely linked to thyroidal pathology. Combined selenium and iodine deficiency appears to be responsible for the formation of myxoedematous cretinism. The proposed mechanism for this is that iodine deficiency results in stimulation of the thyroid by TSH which results in increased production of H_2O_2. In addition, there is a large increase in TSH secretion in the neonatal period which further enhances the production of H_2O_2. Selenium deficiency results in cGPX deficiency and a subsequent increase in accumulation of H_2O_2. The end result is thyroid necrosis and fibrosis (Sunde, 1994; Contempre', Dumont, Denef *et al.*, 1995; Delange, 1996).

In addition to iodine causing suppression of thyroid hormone synthesis (by the Wolff-Chaikoff effect), iodine has effects on thyrocyte growth and immunity. Iodine deficiency leads to increased thyrocyte growth *in vivo* (endemic goitre). In humans with Hashimoto's thyroiditis, the administration of excess dietary iodide may result in hypothyroidism (due to permanent Wolff-Chaikoff inhibition) in greater than 60 per cent of patients (Roti and Vagenakis, 1996). Iodine intake also regulates the pathological type of thyroid disease that occurs in humans. In iodine deficient areas, the percentage of cases of autonomously functioning thyroid nodules is increased compared to that of iodine replete areas (Hay and Morris, 1996). Studies in animals suggest that iodine has an important role in the development of autoimmune thyroid disease. Iodine administration has been shown to induce lymphocytic thyroiditis in hamsters, beagles, mice, certain strains of rats and chickens (Roti and Vagenakis, 1996).

There is, therefore, a strong possibility that dietary factors such as selenium and iodine may play a role in the development of feline toxic nodular goitre.

To date, there have been no reports of the selenium status of the domestic cat. The seemingly marked variation in incidence of feline hyperthyroidism may reflect important differences in environmental and/or dietary factors in cats. As selenium status can dramatically influence thyroid status and expression of certain selenoenzymes such as the deiodinases and TR, we have investigated selenium status in cats as a possible risk factor associated with the development of toxic nodular goitre.

The aims of this study were to:

- examine the expression of selenoenzymes in feline thyrocytes and compare the expression of these enzymes in normal and adenomatous tissue and the response of these selenoenzymes to stimulators of the Ca^{++}/PIP signalling pathway.

- determine the selenium status of cats from areas with allegedly marked differences in the incidence of feline thyrotoxicosis, and to therefore determine if there was any correlation between the apparent incidence of thyrotoxicosis in cats and selenium status in these regions.

7.02 MATERIALS AND METHODS

a) SELENOENZYME EXPRESSION IN THE FELINE THYROCYTE

Feline thyrocytes were harvested and maintained as described in Chapter 2.037. Cells were grown to 80 to 90 per cent confluence, the medium changed and 0.02MBq ^{75}selenium selenite /10ml medium was added to each flask along with the agonist. For each cell preparation, six flasks were used as follows: (1) control 1 (no additions); (2) control 2 (1 per cent v/v DMSO [as DMSO was used for solubilisation of A23187 and PMA]), (3) bTSH (10U/l); (4) calcium ionophore A23187 10^{-6}; (5) PMA 10^{-6}; (6) A23187 10^{-6} plus PMA 10^{-6}. Cells were grown for 48 hours in the ^{75}Se-selenite as previous work in other cell types had shown after this time a steady state had been reached for labelling. Cells were washed four times with EBSS and harvested into approximately 10ml EBSS. The cell suspension was centrifuged for 10minutes at 2000g. The supernatant was discarded and the cell suspension sonicated in 200µl of EBSS. The protein concentration was determined by the Bradford assay (Chapter 2.036) and the sample diluted to 1mg/ml. The sample was then added to 'boiling mix' and heated at 90°C for 10 minutes and subject to SDS-PAGE (Chapter 2.034). The selenoproteins were visualised by autoradiography (Chapter 2.035).

b) SELENIUM STATUS IN CATS FROM AREAS WITH VARYING INCIDENCES OF HYPERTHYROIDISM

i) Collection and transportation of samples

Cats (total n = 50) were recruited from areas with apparent varying incidences of feline hyperthyroidism. These included two areas of high incidence (Edinburgh, U.K. [n = 16]) and Sydney, Australia [n = 14]) and two areas where the disease appears to be less commonly reported (Greve, Denmark [n = 10] and Perth, Western Australia [n = 10]).

Blood was obtained from euthyroid cats being investigated for reasons such as pre-anaesthetic checks and viral tests and also from hyperthyroid cats being bled for diagnostic purposes. Heparinised whole blood (2ml) was immediately frozen at -40°C. A further 2ml of heparinised blood was immediately centrifuged and the plasma and red blood cells were also stored separately at -40°C.

Samples were kept at -40°C until transportation in bulk to Edinburgh was carried out by air freight on dry ice (TNT, Ltd.).

ii) Plasma selenium concentration

Plasma selenium concentration was carried out by the method described in Chapter 2.047.

iii) Plasma and whole blood glutathione peroxidase activity

Plasma and whole blood glutathione peroxidase activity were determined as described in Chapter 2.048. Whole blood glutathione peroxidase activity was corrected for haemoglobin concentration as described in Chapter 2.048.

iv) Statistical analysis

The Kruskall-Wallis test was used to test for statistically significant differences between groups overall. The Mann Whitney U test was used to test for significant differences between two individual areas, with $p < 0.05$ being considered significant.

7.03 RESULTS

a) SELENOPROTEIN EXPRESSION

i) Selenoproteins expressed by feline thyrocytes

Figure 7.01 shows an autoradiograph of an SDS-PAGE demonstrating selenoprotein expression in four FT cell preparations (FT 13, 14, 17 and 20) and four FNG cell preparations (FNG 63, 90, 92 and 95). Both normal and adenomatous feline thyrocytes appeared to express a maximum of 10 major selenoprotein bands but numerous minor bands were also apparent. The selenoproteins expressed in feline thyrocytes that have been previously characterised by our laboratory and others are TR with an Mr of approximately 58KDa, cGPX with an Mr of approximately 24KDa, phospholipid GPX (phGPX) with an Mr of 21 KDa and fatty acid binding protein with an Mr of 14KDa. The identification of TR in human thyrocytes and its regulation by the Ca^{++}/PIP signalling pathway in this laboratory has been recently published (Howie, Arthur, Nicol *et al.*, 1998).

The expression of certain selenoenzymes was variable between FT and FNG cells. One adenomatous preparation showed a markedly different selenoenzyme profile to any other feline thyrocyte preparation (FNG63). This preparation showed an overexpression of TR, and a loss the 14KDa band observed in all other preparations. TR expression in two adenomatous preparations (FNG90 and FNG92) was similar to that of four normal glands (FT17, FT14, FT13 and FT20). TR expression was high in two adenoma preparations (FNG63 and FNG95). Normal thyrocyte preparations showed some variation in selenoprotein expression such as differences in expression of the 21KDa band and TR (Figure 7.01). Both normal and adenomatous feline thyrocytes expressed two bands that migrated closely in the 58KDa region and may subsequently prove to be different isoforms of TR.

ii) Type 1 deiodinase (IDI) in feline thyrocytes

Figures 7.01, 7.02 and 7.03 demonstrate the selenoprotein expression from a number of normal and adenomatous feline thyrocyte preparations with the cell lines FRTL-5 and HepG2 cells as controls. Both of the two control cell line types show the expression of a 29KDa selenoenzyme

previously characterised as IDI (Chapter 3). There is no evidence of a similar IDI band in any of the feline thyrocyte preparations, even after the addition of bTSH (10U/l) (Figure 7.04).

iii) Thioredoxin reductase (TR) expression in feline thyrocytes

In both FT and FNG cells, bTSH resulted in no change in the expression of TR (Fig. 7.04). The calcium ionophore A23187, resulted in variable changes in TR expression from no change in expression (FNG90 and FT13) to increased expression in the majority of both normal (FT14, 15, 17 and 20) and adenomatous cell preparations (FNG63, 92 and 95). TR clearly appeared to be overexpressed in two FNG preparations (FNG63 and FNG95).

The phorbol ester, phorbol-12-myristate-13-acetate (PMA) produced a more convincing up-regulation of TR in both normal and adenomatous feline thyrocytes. The addition of A23187 and PMA in combination resulted in a further enhancement of this expression in some FT and FNG preparations studied (Figs. 7.02 and 7.03). This pattern of regulation by A23187 and PMA has also been previously demonstrated in sheep (Fig. 7.05) and human thyrocytes in this laboratory (Beckett, Howie, Nicol *et al.,* 1998).

b) SELENIUM STATUS IN CATS FROM AREAS WITH VARYING INCIDENCES OF HYPERTHYROIDISM.

i) Clinical data for the 50 cats studied

The clinical data and thyroid status for the 50 cats is presented in Table 7.01.

Table 7.01: Clinical data for the 50 cats investigated from areas with varying incidences of hyperthyroidism.

Cat	Age (years)	Breed	Sex	Euthyroid / hyperthyroid
Greve, Denmark				
1	6	Persian	MN	Euthyroid
2	11	Persian	MN	Euthyroid
3	8	Persian	MN	Euthyroid
4	6	British Shorthair	MN	Euthyroid
5	14	Oriental	FN	Euthyroid
6	6	DSH	MN	Euthyroid
7	7	DSH	FN	Euthyroid
8	8	DSH	FN	Euthyroid
9	6	DSH	MN	Euthyroid
10	8	DSH	MN	Euthyroid
Perth, Western Australia				
11	5	Siamese	MN	Euthyroid
12	3	Persian	ME	Euthyroid
13	1	Persian	FE	Euthyroid
14	1	Persian	ME	Euthyroid
15	1	Persian	FE	Euthyroid
16	9	Persian	FE	Euthyroid
17	10	Persian	FE	Euthyroid
18	3	Burmese	MN	Euthyroid
19	10	Burmese	MN	Euthyroid
20	3	Persian	ME	Euthyroid
Sydney, Eastern Australia				
21	12	DSH	MN	Hyperthyroid
22	18	DSH	FN	Hyperthyroid
23	9	DSH	MN	Euthyroid
24	6	DSH	FN	Euthyroid
25	12	DSH	FN	Euthyroid
26	14	DSH	MN	Hyperthyroid
27	18	DSH	MN	Euthyroid
28	6	DSH	MN	Euthyroid
29	5	DSH	MN	Euthyroid
30	8	DSH	MN	Euthyroid
31	2	DSH	FN	Euthyroid
32	12	DSH	FN	Euthyroid
33	4	Siamese	MN	Euthyroid
34	6	DSH	FN	Euthyroid

Table 7.01 (continued): Clinical data for the 50 cats investigated from areas with varying incidences of hyperthyroidism.

Edinburgh, U.K.

35	12	DSH	MN	Hyperthyroid
36	10	DSH	FN	Euthyroid
37	12	Siamese	MN	Euthyroid
38	10	DSH	MN	Euthyroid
39	8	DSH	FN	Euthyroid
40	8	DSH	MN	Euthyroid
41	14	DLH	MN	Euthyroid
42	7	DSH	MN	Euthyroid
43	10	DSH	FN	Euthyroid
44	9	DSH	FN	Euthyroid
45	12	DSH	FN	Euthyroid
46	11	DSH	MN	Euthyroid
47	7	DSH	MN	Euthyroid
48	16	DSH	FN	Hyperthyroid
49	14	DSH	MN	Hyperthyroid
50	12	DSH	MN	Hyperthyroid

DLH, Domestic longhair; DSH, Domestic shorthair; FE, Entire female; FN, Ovariohysterectomised female; ME, Entire male; MN, Castrated male. NB: The numbers identifying the cats in this table do not represent the cases studied in the selenoprotein expression section.

ii) PLASMA SELENIUM CONCENTRATIONS.

Data for plasma selenium concentrations from all cats are presented in Table 7.02 and graphically in Figure 7.06. There was no significant difference between the groups overall ($p = 0.1650$). Further, there was no significant difference between any two individual groups (e.g.: Greve and Perth) ($p > 0.05$ in every case).

iii) WHOLE BLOOD GLUTATHIONE PEROXIDASE ACTIVITY.

Data for whole blood GPX activity are presented in Table 7.03 and graphically in Figure 7.07. There was no significant difference between the groups overall ($p = 0.0800$). However, Edinburgh cats had significantly lower whole blood glutathione peroxidase activity than Greve cats ($p = 0.0481$) but there was no significant difference between any other two individual groups ($p > 0.05$).

iv) PLASMA GLUTATHIONE PEROXIDASE ACTIVITY.

Data for plasma GPX activity are presented in Table 7.04 and graphically in Figure 7.08. There was no significant difference between the groups overall ($p = 0.0510$). However, Edinburgh cats had significantly lower plasma GPX activity than Greve cats ($p = 0.0112$), but there was no significant difference between any other two individual groups ($p > 0.05$).

v) CORRELATION BETWEEN PLASMA SELENIUM CONCENTRATIONS AND WHOLE BLOOD AND PLASMA GLUTATHIONE PEROXIDASE ACTIVITY.

The was no correlation between plasma selenium concentration and whole blood glutathione peroxidase activity in the 50 cats ($p = 0.7380$) (Figure 7.09). There was no correlation between plasma selenium concentration and plasma glutathione peroxidase activity in the 50 cats ($p = 0.0820$) (Figure 7.10).

vi) PLASMA SELENIUM STATUS AND HYPERTHYROIDISM

Of the 50 cats studied, seven were thyrotoxic (three from Sydney and four from Edinburgh). The range of plasma selenium concentrations of these seven cats was 0.3100 to 0.5980ppm/ml

(mean 0.4397 ± 0.1014). There was no significant difference in plasma selenium concentration ($p = 0.4389$), whole blood glutathione peroxidase activity ($p = 0.4337$) or plasma glutathione peroxidase activity ($p = 0.9251$) between the euthyroid and hyperthyroid cats. There was no significant difference in plasma selenium concentration ($p = 1.0000$), whole blood glutathione peroxidase activity ($p = 0.2772$) or plasma glutathione peroxidase activity ($p = 0.3496$) between the euthyroid and hyperthyroid cats from Edinburgh. There was no significant difference in plasma selenium concentration ($p = 0.4069$), whole blood glutathione peroxidase activity ($p = 0.8026$) or plasma glutathione peroxidase activity ($p = 0.7642$) between the euthyroid and hyperthyroid cats from Sydney.

Table 7.02: Plasma selenium status (ppm/ml) in cats from areas with varying incidences of hyperthyroidism.

Area	Median	Min	Max	Q1	Q3
Greve	0.5120	0.3320	0.6220	0.4352	0.5847
Perth	0.4100	0.3120	0.5720	0.3460	0.5180
Sydney	0.5190	0.3850	0.6610	0.4115	0.5545
Edinburgh	0.4230	0.3100	0.6870	0.3760	0.5050
All cats	**0.4550**	**0.3120**	**0.6870**	**0.4050**	**0.5425**

Table 7.03: Whole blood glutathione peroxidase activity (μM NADPH oxidised per gm haemoglobin) in cats from areas with varying incidences of hyperthyroidism.

Area	Median	Min	Max	Q1	Q3
Greve	404.9	230.7	587.0	312.0	493.1
Perth	409.0	236.0	492.5	347.1	404.4
Sydney	356.5	153.1	522.8	291.0	425.0
Edinburgh	320.9	200.9	427.0	286.7	377.0
All cats	**354.3**	**153.1**	**587.0**	**304.1**	**449.0**

Table 7.04: Plasma glutathione peroxidase activity (μM NADPH oxidised per gm haemoglobin) in cats from areas with varying incidences of hyperthyroidism.

Area	Median	Min	Max	Q1	Q3
Greve	8.6	6.20	11.40	7.25	10.60
Perth	6.9	5.30	16.32	6.30	9.65
Sydney	7.6	4.50	11.70	6.33	9.38
Edinburgh	6.0	4.70	13.30	5.30	6.80
All cats	**7.30**	**4.50**	**16.32**	**6.10**	**9.20**

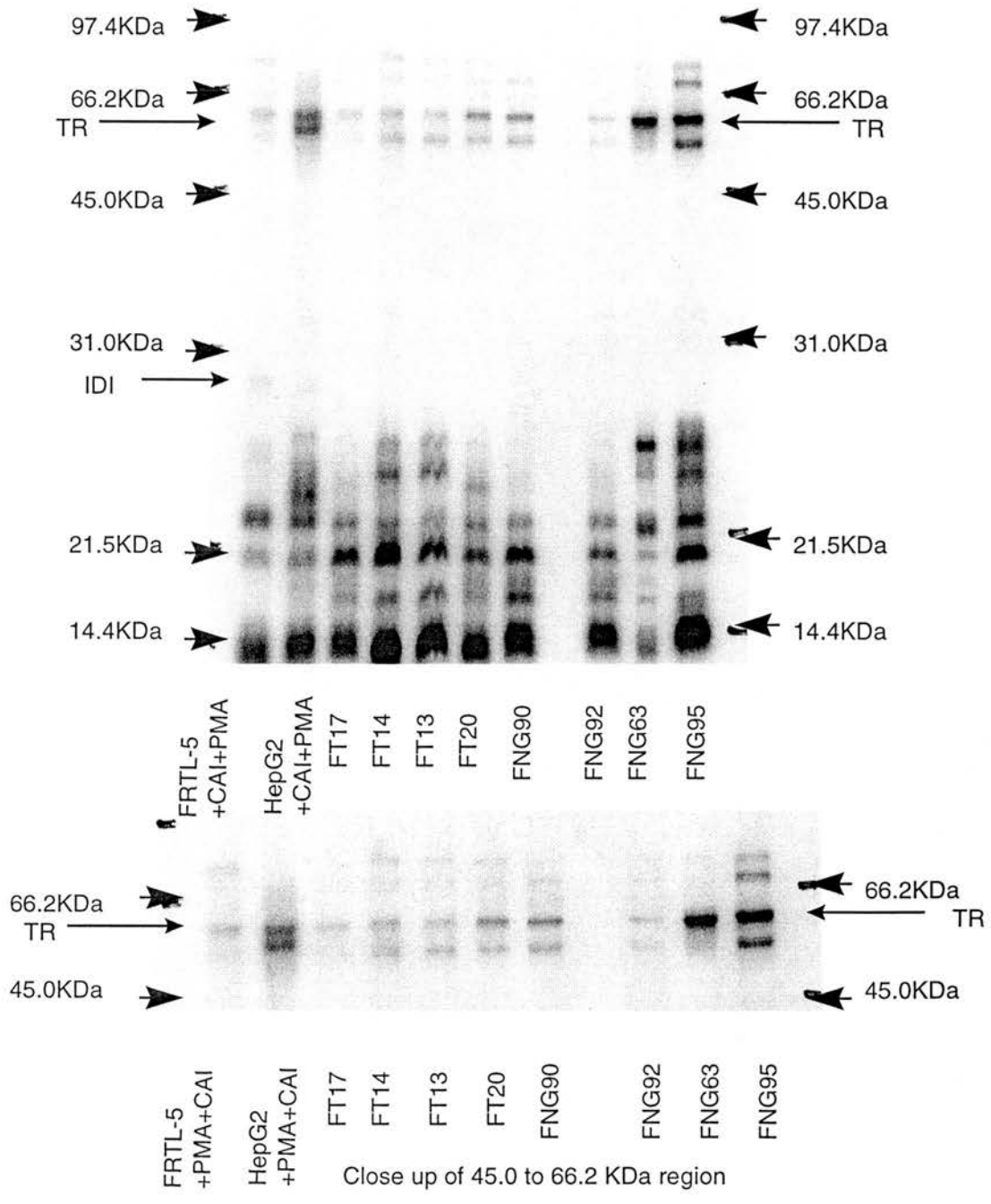


Figure 7.01: Autoradiograph of SDS-PAGE of four normal and four adenomatous feline thyrocyte (^{75}Se) selenoprotein profiles with FRTL-5 and HepG2 cells as controls grown under basal conditions. FNG, feline adenomatous cells; FT, normal feline thyrocytes; GPX, intracellular glutathione peroxidase; IDI, type I iodothyronine deiodinase; TR, thioredoxin reductase.

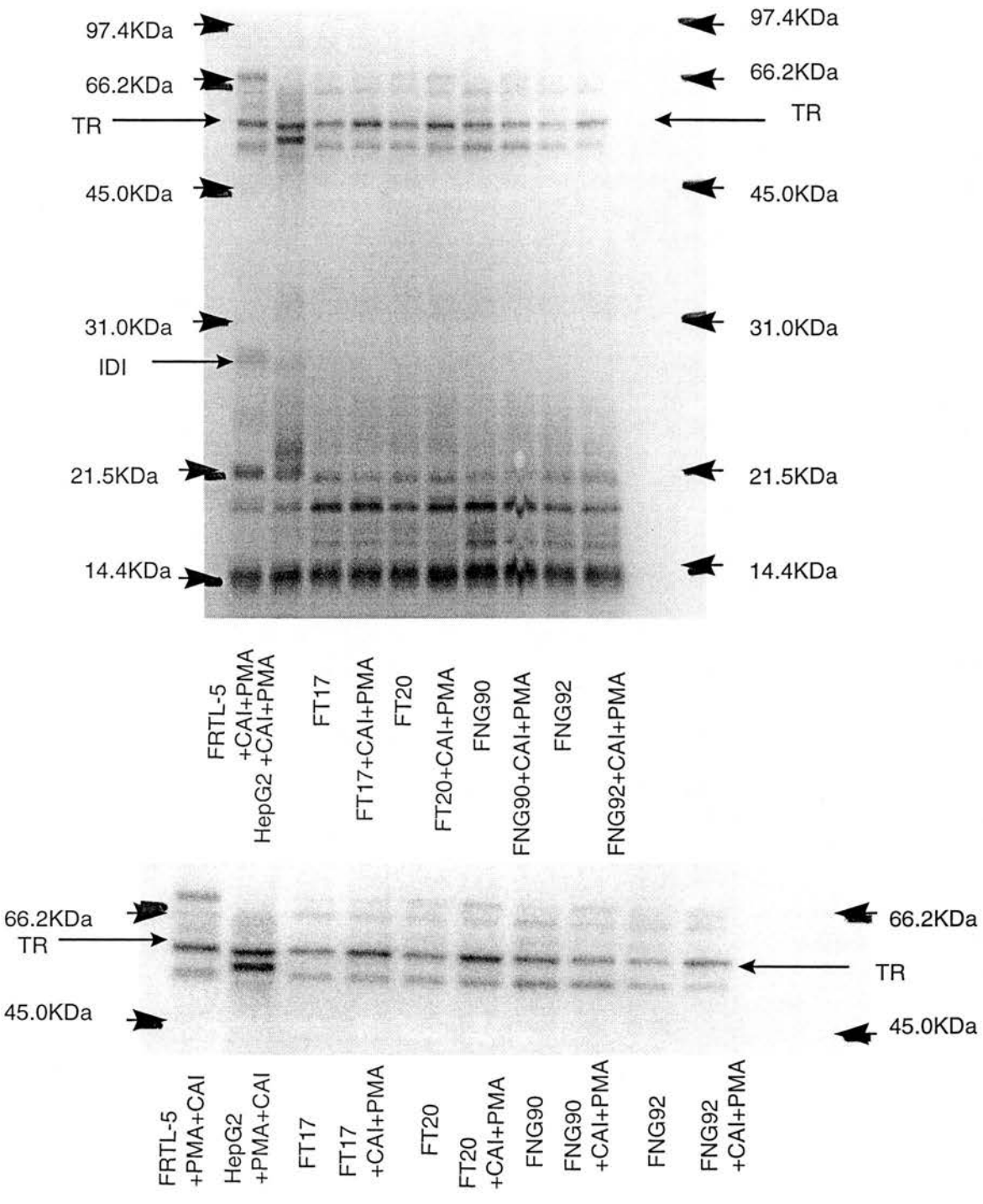
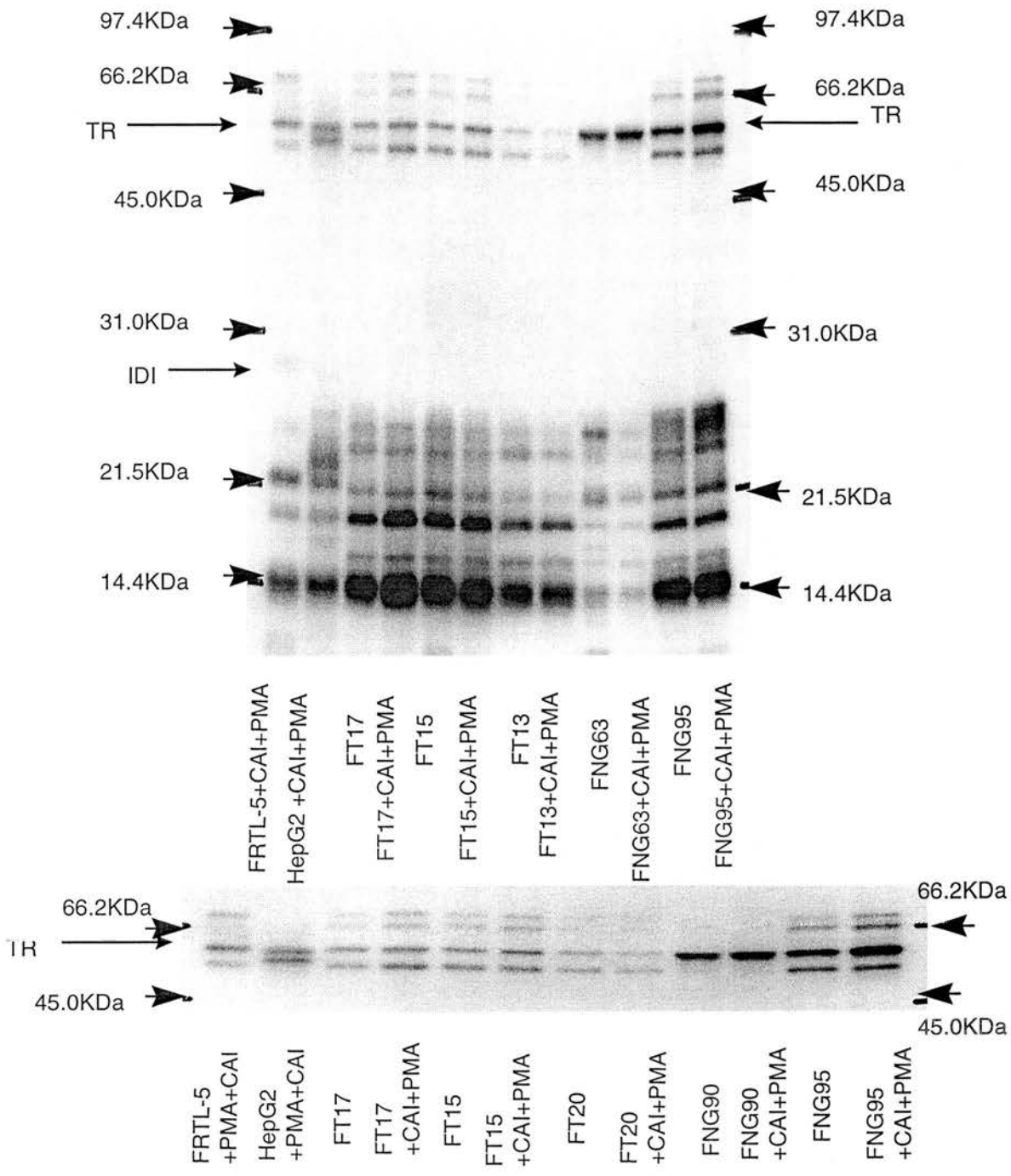


Figure 7.02: Autoradiograph of SDS-PAGE of two normal and two adenomatous feline thyrocyte (^{75}Se) selenoprotein profiles with FRTL-5 and HepG2 cells as controls. CAI, calcium ionophore 10^{-6} (A23187); FNG, feline adenomatous cells; FT, normal feline thyrocytes; GPX, intracellular glutathione peroxidase; IDI, type I iodothyronine deiodinase; PMA, phorbol ester 10^{-6}; TR, thioredoxin reductase.



Close up of 45.0 to 66.2 KDa region

Figure 7.03: Autoradiograph of SDS-PAGE of three normal and two adenomatous feline thyrocyte (^{75}Se) selenoprotein profiles with FRTL-5 and HepG2 cells as controls. CAI, calcium ionophore 10^{-6} (A23187); FNG, feline adenomatous cells; FT, normal feline thyrocytes; GPX, intracellular glutathione peroxidase; IDI, type I iodothyronine deiodinase; PMA, phorbol ester 10^{-6}; TR, thioredoxin reductase.

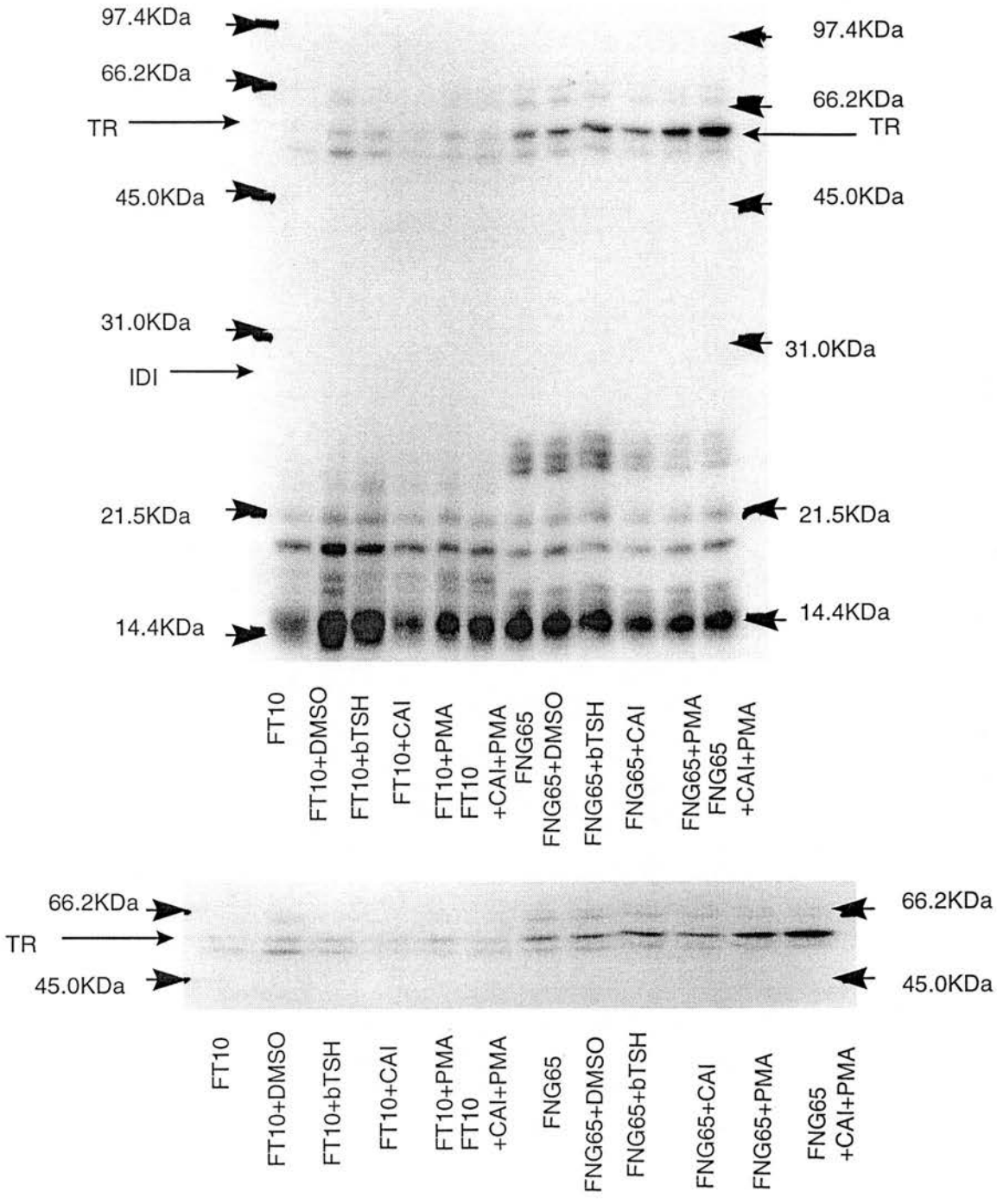


Figure 7.04: Autoradiograph of SDS-PAGE of one normal feline thyrocyte preparation (FT10) and one adenomatous feline thyrocyte preparation (FNG65) (^{75}Se) selenoprotein profiles. bTSH, bovine thyrotropin stimulating hormone (10U/l); CAI, calcium ionophore 10^{-6} (A23187); FNG, feline adenomatous cells; FT, normal feline thyrocytes; GPX, intracellular glutathione peroxidase; IDI, type I iodothyronine deiodinase; PMA, phorbol ester 10^{-6}; TR, thioredoxin reductase.

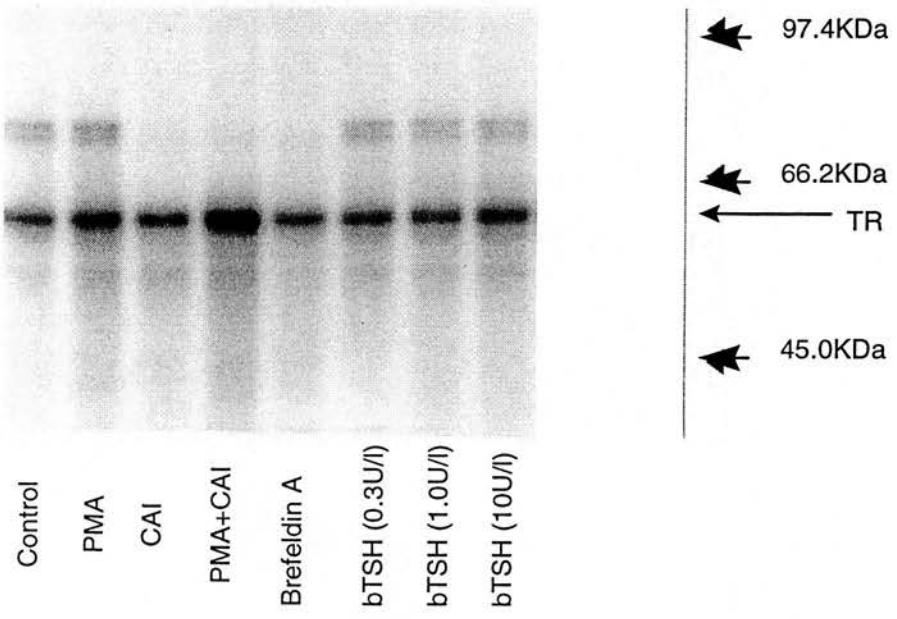


Figure 7.05: Autoradiograph of SDS-PAGE of sheep thyrocytes (^{75}Se) selenoprotein profiles. bTSH, bovine thyrotropin stimulating hormone; CAI, calcium ionophore 10^{-6} (A23187); PMA, phorbol ester 10^{-6}; TR, thioredoxin reductase.

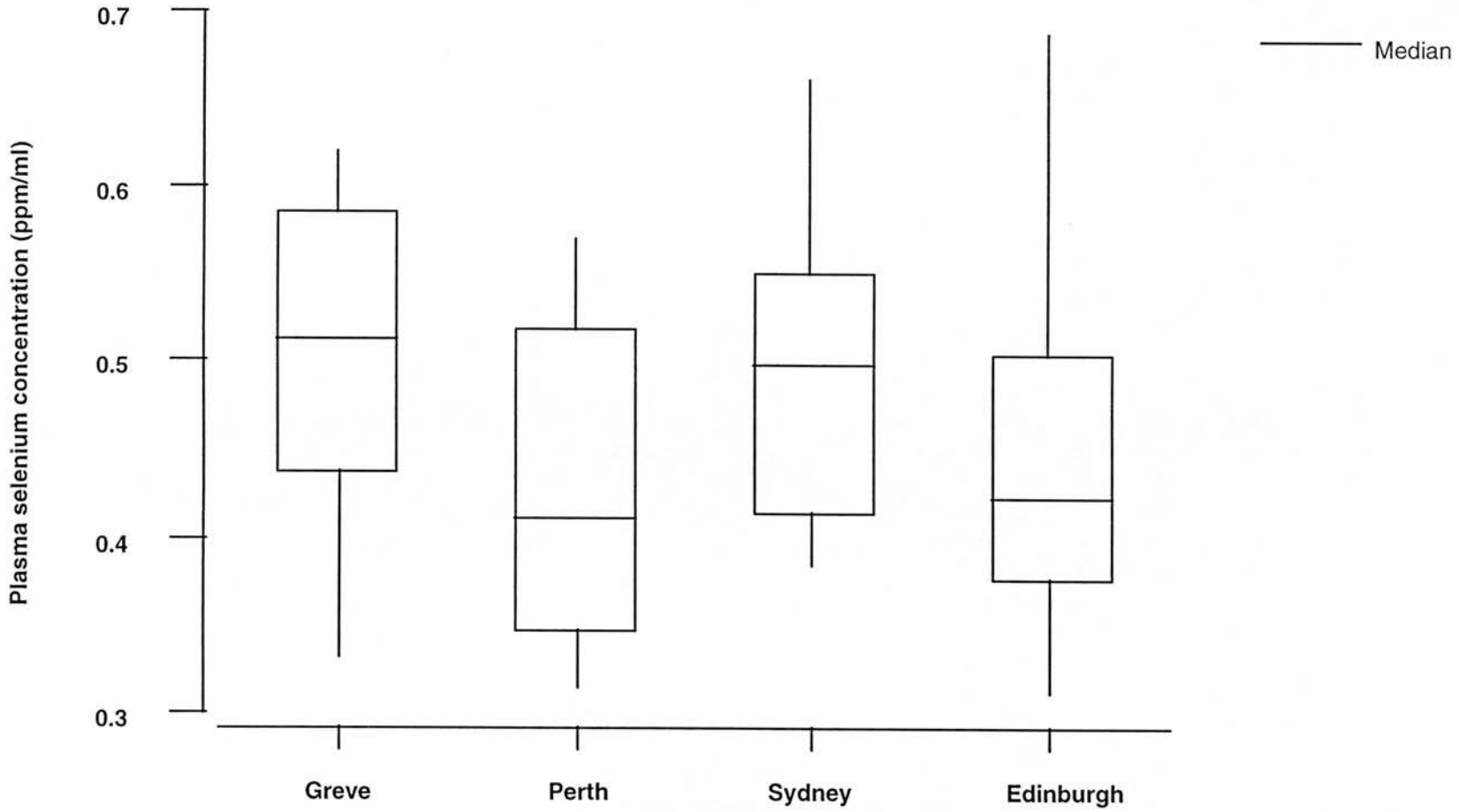


Figure 7.06: Plasma selenium concentrations (ppm/ml) in cats from areas with varying incidences of hyperthyroidism. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3). Whiskers define the range of the data.

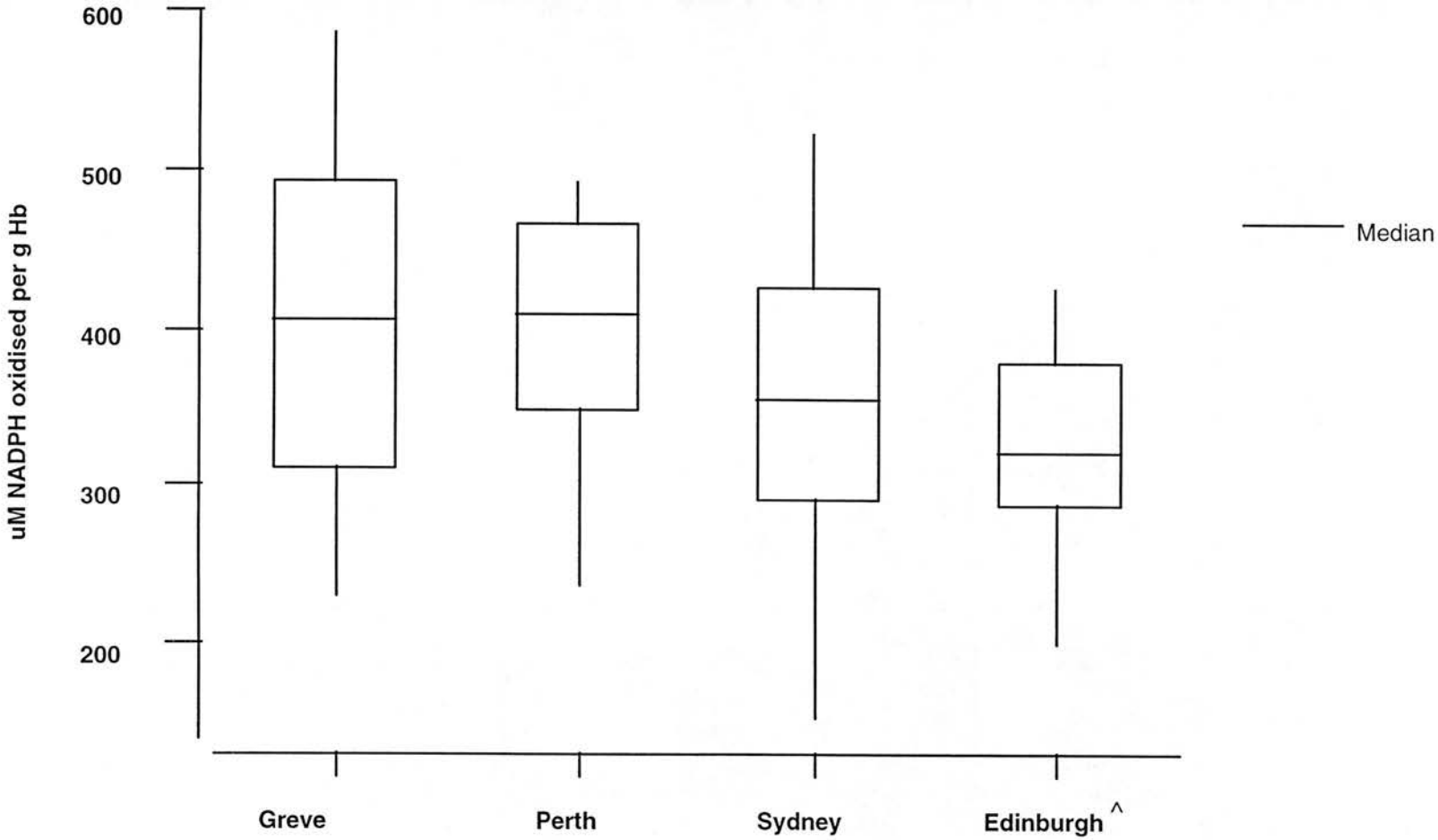


Figure 7.07: Whole blood GPX activity in cats from areas with varying incidnences of hyperthyroidism. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3). Whiskers define the range of the data. $p < 0.05$^.

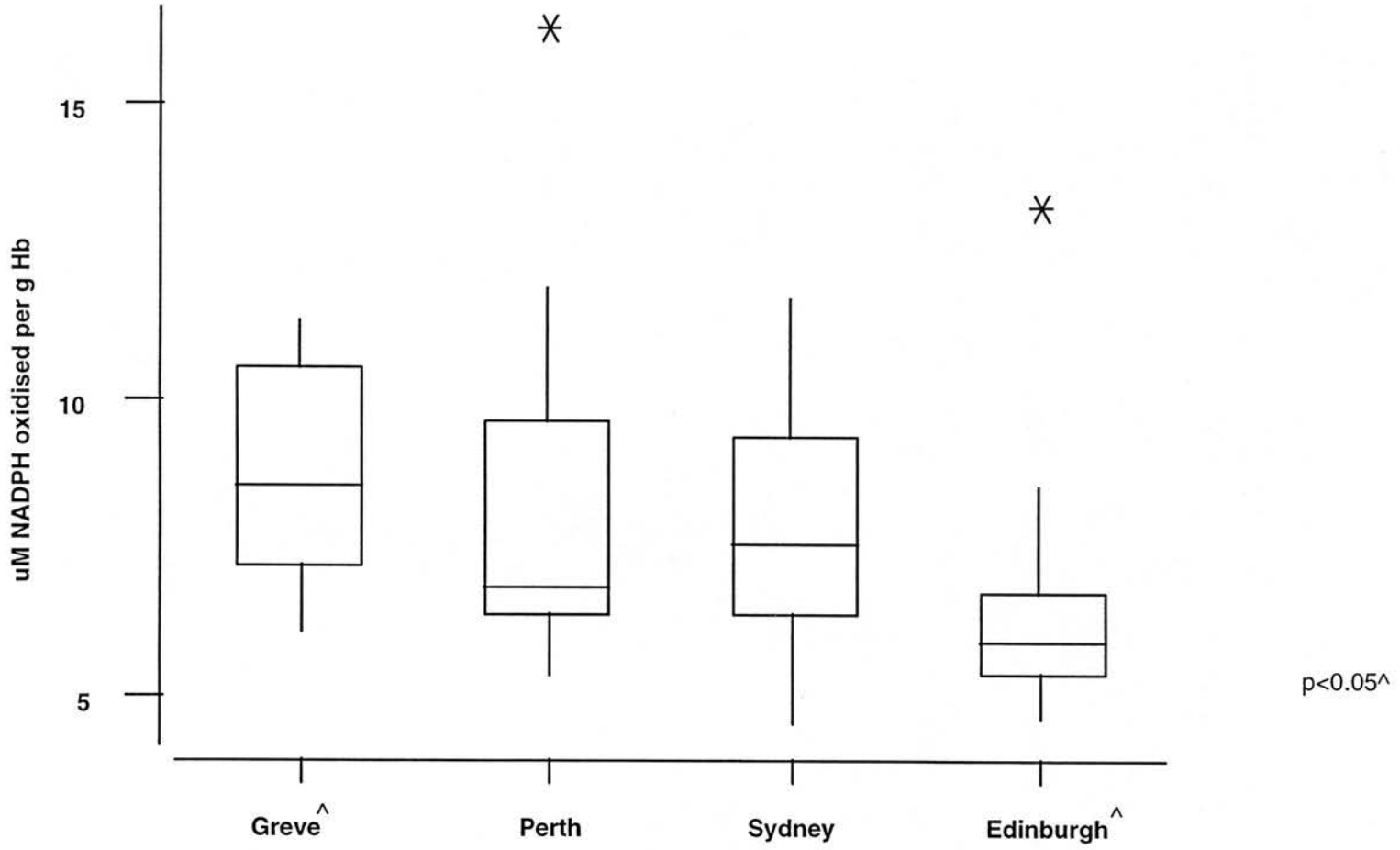


Figure 7.08: Plasma GPX activity in cats from areas with varying incidnences of hyperthyroidism. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3). Whiskers define the range of the data. p< 0.05^. Outliers are represented by *.

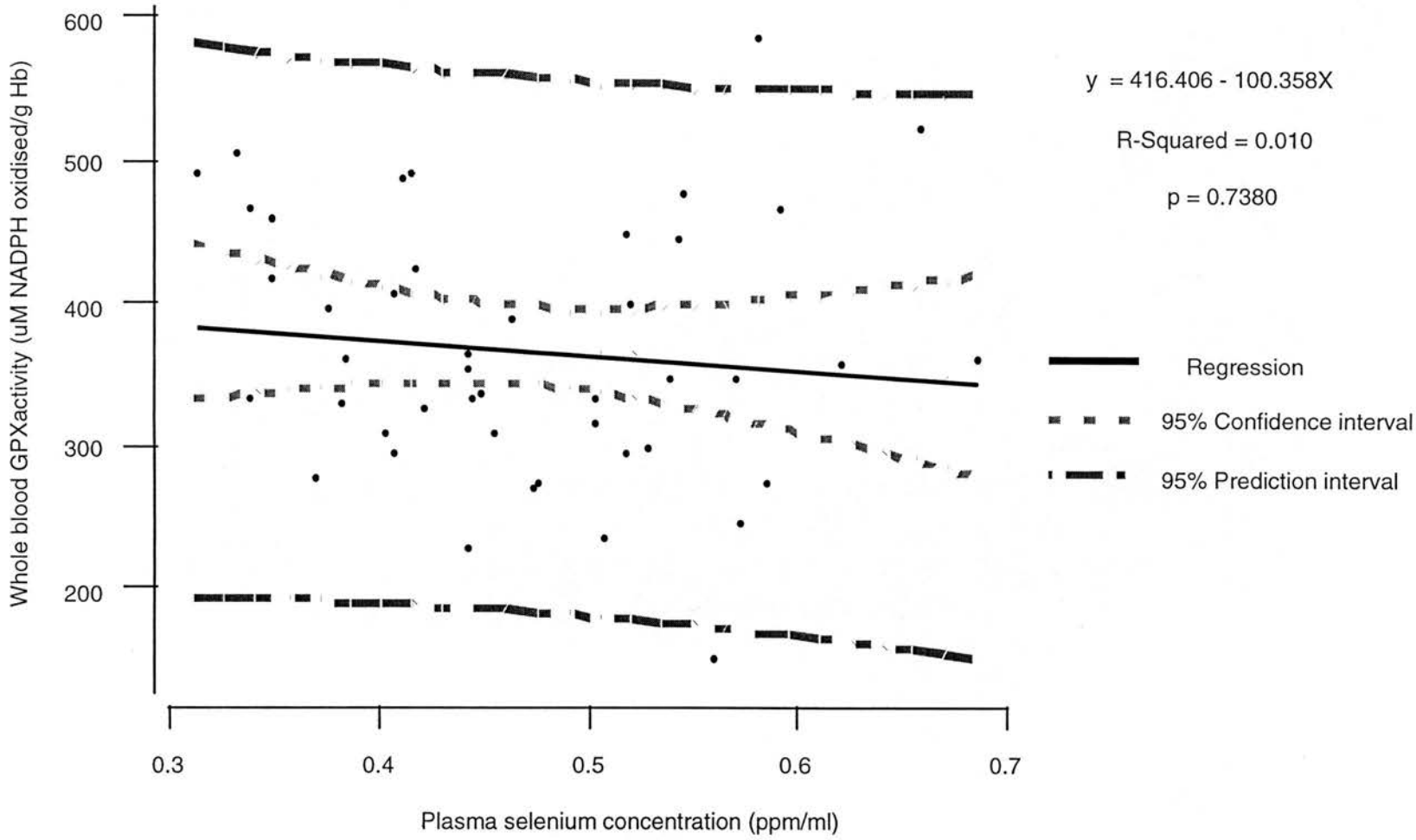


Figure 7.09: Regression analysis of plasma selenium concentration vs whole blood glutathione peroxidase activity.

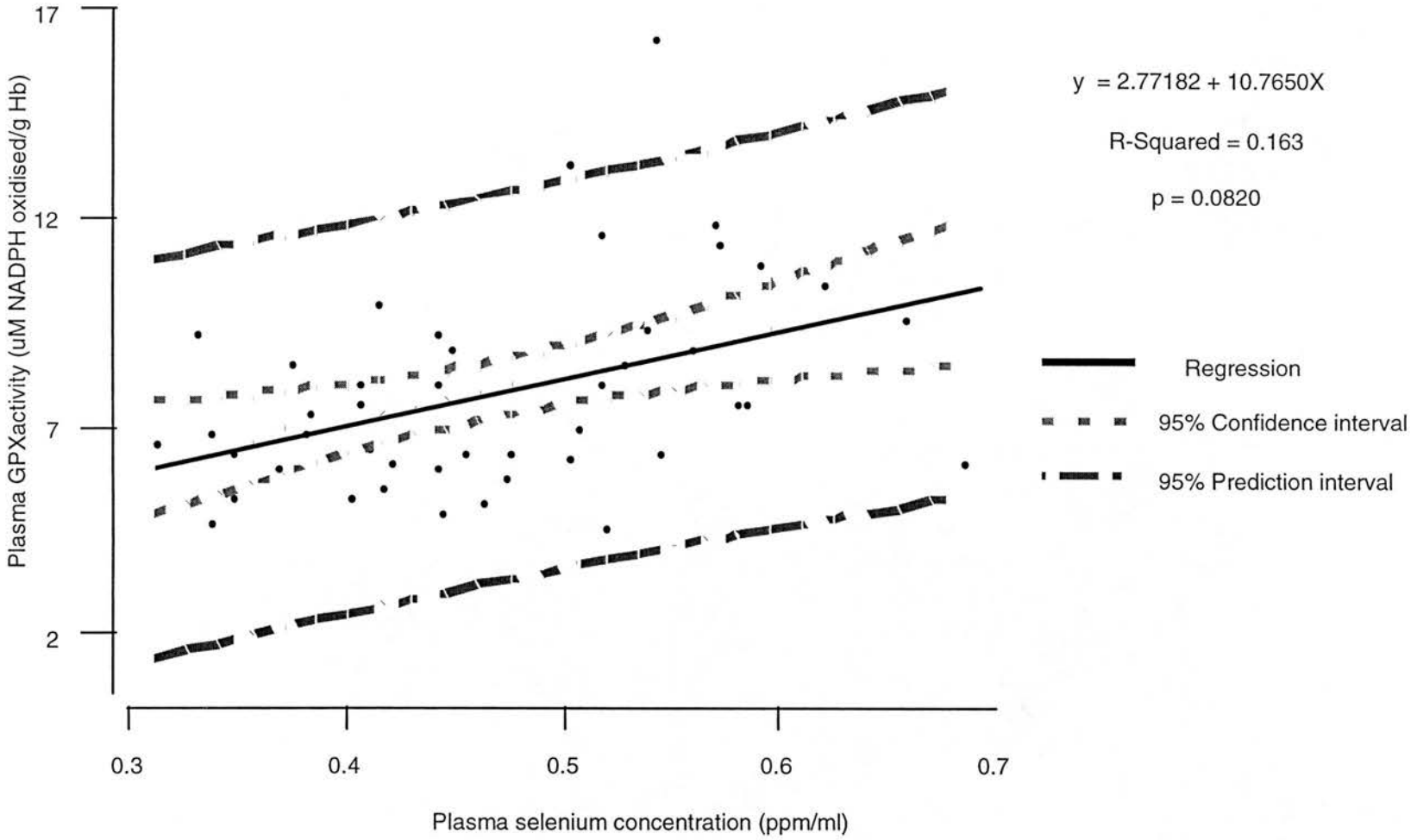


Figure 7.10: Regression analysis of plasma selenium concentration vs plasma glutathione peroxidase activity.

7.04 DISCUSSION

The human thyroid gland contains higher concentrations of selenium than any other human organ per gram of tissue (Aaseth, Frey, Glaattre *et al.,* 1990). When there is adequate iodine supply, the rate limiting step in thyroid hormone synthesis is the amount of H_2O_2 present at the apical membrane of the thyrocyte. Degradation of H_2O_2 in the follicular lumen is regulated by E-GPX and intracellular GPXs detoxify H_2O_2 to produce H_2O and thus protects the thyrocyte from its harmful oxidant effects. It has been suggested that E-GPX is an important regulator of thyroid hormone synthesis in the human thyrocyte (Howie, Walker, Akesson *et al.,* 1995). In humans, stimulation of the PIP pathway increases H_2O_2 synthesis and decreases secretion of E-GPX into the follicular lumen thus leading to increased H_2O_2 concentrations into this space. In addition, thyroid hormone deiodination is also influenced by selenium status. Hepatic and renal deiodinase type I are down-regulated in selenium deficiency, whilst thyroidal IDI (if expressed) is up-regulated. These observations suggest a hierarchy of selenium supply to specific tissues and between selenoenzymes within a tissue. As the present work has demonstrated in Chapter 3, the cat is unique as an obligate carnivore in that it lacks the expression of thyroidal IDI. The marked geographical distribution of feline hyperthyroidism makes the investigation of dietary factors such as selenium status in cats of particular interest. In addition, selenoenzymes such as TR have been shown to regulate growth of a number of cell types and may have important roles in the development of toxic nodular goitre in the cat. The thioredoxin redox system plays an important role in regulating cell growth and death (Gasdaska, Berggren, Berry *et al.*, 1999).

Feline thyrocytes (FT and FNG) appear to express at least 10 major selenoproteins. The selenoproteins of particular interest are cGPX (24KDa) and TR (58KDa). The lack of expression of IDI (29KDa) confirms data presented in Chapter 3, i.e. that cats are unique among carnivores/omnivores previously studied (Beckett, Beech, Nicol *et al.,* 1993). The selenoprotein profiles of normal thyrocyte preparations varied between each other as did the selenoprotein profiles from the adenomatous thyrocyte preparations. TR appeared to be more commonly overexpressed in adenomatous cells compared to normal feline thyrocytes. TR has been demonstrated to be a 58KDa protein and is expressed in many cell lines and tissues. Our laboratory has confirmed TR expression in human thyrocytes (Howie, Arthur, Nicol *et al.*, 1998) and HepG2 cells by selenoprotein labelling and Western blotting with HepG2 cells being used as a control.

A number of TR enzymes have been sequenced (Gasdaska, Gasdaska, Cochran *et al.*, 1995; Gladyshev, Jeang and Stadtman, 1996; Tamura and Stadtman, 1996; Zhong, Arner, Ljung *et al.*, 1998). Recently, the possibility that TR occurs in multiple forms has been raised. In the present study, feline thyrocytes expressed two bands that migrate closely in the 58KDa region, further characterisation (including cloning and function expression in other cell types) may prove these to be two different isoforms of TR.

The regulation of TR expression by PMA and calcium ionophore (A23187) was also variable among preparations. PMA is an activator of protein kinase C *in vivo* and *in vitro* and potentiates forskolin induced cAMP formation (Alberts, Bray, Lewis *et al.*, 1989). The calcium ionophore (A23187) is used to increase intracellular Ca^{++} concentrations and uncouple oxidative phosphorylation (Alberts, Bray Lewis *et al.*, 1989). In our laboratory, both human and sheep thyrocytes show an increased expression of TR with PMA alone (Beckett, Howie, Nicol *et al.,* 1998). A23187 also increased TR expression in human thyrocytes, but not in sheep thyrocytes (Figure 7.0). The combined addition of PMA and A23187 in FRTL-5 cells resulted in increased TR expression, but PMA or A23187 alone did not (Beckett, Howie, Nicol *et al.,* 1998). TSH produced no noticeable change in TR expression in any cell type. These observations are similar to those in the present study of feline thyrocytes. TR showed no change in expression when A23187 was used alone (as in sheep thyrocytes), a variable increase in expression when PMA was used alone and a more consistent increase in expression when PMA/A23187 were used in combination. There were two adenomatous feline thyrocyte preparations with high basal expression of TR (FNG63 and FNG95). It is not surprising that the expression of TR and other selenoproteins is variable amongst adenoma preparations as there is a heterogenous mixture of cells. What is perhaps more surprising is the variable expression of selenoenzymes in 'normal' feline thyrocyte preparations. The reasons for this are unknown, but may include different selenium status, breed, sex and age related differences and variable states of thyroid hormone production (i.e.: active glands versus more quiescent glands).

TR has been shown to be associated with the regulation of growth in certain cell lines (Gallegos, Gasdaska, Taylor *et al.,* 1996). TR is secreted by cells and may, therefore, act as an autocrine growth factor (Berggren, Gallegos, Gasdaska *et al.,* 1996). A number of human primary cancers (such as those from lung, colon, cervix and liver) have also been shown to over-express thioredoxin and transfection with thioredoxin can increase tumour growth and inhibit apoptosis

(Gasdaska, Berggren, Berry *et al.*, 1999). It may be that in some adenomas TR acts as a growth stimulus. It would be interesting to determine the growth characteristics of feline thyroid adenomas, and correlate this with TR expression to determine if there is an association between TR and growth in this feline disease. The patterns of selenoenzyme expression in feline thyrocytes and the possible role of TR in the regulation of growth of feline thyrocytes and subsequent development of adenomas warrants further investigation.

Because of the marked influence that selenium status has on thyroid hormone synthesis and metabolism, and that certain selenoenzymes (e.g. TR) have effects on the growth of cells, we compared the selenium status in cats from four different regions. Anecdotal evidence suggests that feline thyrotoxicosis is a less common disease of cats in Greve, Denmark and Perth, Western Australia. For this reason, we chose to examine the selenium status of cats from two areas with apparently high incidence of hyperthyroidism (Edinburgh, U.K. and Sydney, Eastern Australia) and two areas with seemingly low incidences of hyperthyroidism (Greve, Denmark and Perth, Western Australia).

The present study demonstrates there was no significant difference in plasma selenium concentrations between the four areas overall. Markers of selenium activity (whole blood, or plasma glutathione peroxidase) were also not significantly different between the four groups. Although the population of Greve cats studied had statistically significantly higher plasma and whole blood glutathione peroxidase than the Edinburgh samples, the difference is likely to be of little clinical significance.

The most surprising finding was that cats had approximately 10 times the concentrations of plasma selenium and whole blood GPX activity of selenium replete rats and humans (Koller and Exon, 1986; Arthur, Nicol, Hutchinson *et al.,* 1990; Foster and Sumar, 1997). Whilst no studies have been reported on selenium requirements of cats, National Research Council (NRC) requirements for cats are used to calculate selenium concentrations of cat foods by at least one major pet food company (Waltham, personal communication 1999). Selenium content of cat foods from Waltham are stated as being in the range of 10 to 40 µg/400Kcal ME. The stated calorific requirement of cats (70 to 90 kcal/kg/day) should, therefore, lead to an average daily intake of approximately 7 to 36µg of selenium per cat, per day. This selenium intake

(approximately 1.75 to 9µg/kg/day) is far higher than that recommended for the average human (55 and 70µg/day in adult women and men respectively, approximately 1µg/kg) (Neve, 1998) and greater than that which is thought to be consumed daily by humans in the U.K. (20 to 300µg/day; approximating to 0.3 to 4.5µg/kg/day)(Foster and Sumar, 1995). The fact that cats have approximately 10 times the serum selenium concentration of humans may be the result of increased dietary intake, the ability of cats to absorb selenium and store it more efficiently than other animals, or that they are less able to excrete it. The present study appears to show that the calculation of selenium requirements for cats may be overestimated.

Our studies showed no difference between selenium status in euthyroid and hyperthyroid cats. This is in contrast to published human studies which demonstrate that the selenium status in Graves' disease and multinodular goitre patients are lower than that found in euthyroid age-matched controls (Beckett, Peterson, Choudhury *et al.*, 1991; Bellisola, Cinque, Galassini *et al.,* 1998). It may be that the cats we investigated had such high selenium intakes that their selenium states could not be affected by the thyroid status. Alternatively it may be the population we selected to study. Our hyperthyroid and euthyroid cats were not age-matched and this may have affected the results.

No papers and only two abstracts have been published reporting the bioavailability of selenium in dog and cat foods. These abstracts suggest that in a bioavailability assay, selenium appears to be poorly bioavailable in dog and cat food (Wedekind, Cowell and Combs, 1997; Wedekind, Bever and Combs, 1997). From our data, this assumption, at least for cats, appears unlikely.

The fact that our data shows that there is no correlation between plasma selenium concentrations nor either whole blood or plasma glutathione activities, strongly suggests that most cats are selenium replete. Correlation between plasma selenium concentrations and glutathione peroxidase activity is apparent only when plasma or selenium concentrations are low. At high intakes of selenium, GPX activity in whole blood plateaux but plasma selenium concentrations continue to rise (Foster and Sumar, 1995).

Whilst the incidence of feline hyperthyroidism is reported to have increased since it was first recognised it is difficult to establish what separate roles of increased awareness, diagnostic ability, longevity and numbers of cats, or the possibility of increased exposure to goitrogens, have played in this increased incidence. It does appear, however, that the increase in incidence goes beyond what might be reasonably expected from the increased awareness of the disease.

An important question still remains to be answered: is selenium involved in the pathogenesis of feline thyrotoxicosis? It may appear that as the plasma selenium concentrations from areas with variable incidences are not significantly different from each other, that this trace element has little involvement in the pathogenesis of this disease. There may however be other important factors which do not allow the development of feline hyperthyroidism in Greve or Perth. Risk factors that have been demonstrated in one epidemiological survey included the use of flea-sprays, pesticides/herbicides, living indoors and eating mainly tinned food (Scarlett, Moise and Rayl., 1988). Another important factor may be the possible variability in the longevity of cats between the regions. More extensive studies on the longevity of cats and the actual incidence of feline hyperthyroidism from these areas, may help to explain the marked geographical boundaries of feline hyperthyroidism and elucidate risk factors.

In addition, the combined roles of selenium and iodine in thyroid physiology and growth of thyrocytes may be an important area of investigation in the pathogenesis of feline hyperthyroidism. Certainly, these two trace elements interact in the development of thyroid disease in humans, for example, combined selenium and iodine deficiency in areas of endemic goitre appears to alter the ratio in favour of myxoedematous cretinism (Delange, 1996). Iodine intake has marked effects on thyroid hormone production, thyrocyte growth, thyroid immunity (Roti and Vagenakis, 1996) and, in addition, regulates the pathological type of thyroid disease that occurs in humans. In iodine deficient areas, the percentage of cases of autonomously functioning thyroid nodules is increased compared to that of iodine replete areas (Hay and Morris, 1996).

The iodine content of cat food has been shown to be very variable. Mumma, Rashid, Shane *et al.* (1986) showed that it could contain up to 10 times the recommended daily intake, possibly due to the inclusion of thyroid glands from slaughtered animals. In another study, Johnson, Ford,

Tartellin *et al.* (1992) reported that two brands of commercial cat food contained excess amounts of iodine whilst nine contained less than the recommended intake. They speculated that a wide variation in iodine intake may be responsible for thyroid dysfunction in the cat. One major pet food company regulates iodine content in cat foods to provide adult cats with approximately 0.1 to 4.5mg of iodine per cat per day (personal communication 1999, Waltham). It is certainly possible that high a selenium intake may modify selenoprotein growth factors such as TR and this, coupled with variable iodine intake which is also known to affect the growth of thyrocytes and local thyroid immunity, may lead to the formation of toxic nodular goitre. Long term studies investigating the selenium and iodine status of cats would be of very significant interest.

8.00 THE USE OF PROPRANOLOL AND POTASSIUM IODATE IN THE PRESURGICAL MANAGEMENT OF HYPERTHYROID CATS

8.01 INTRODUCTION

The induction of euthyroidism by prior short-term medical therapy dramatically reduces peri-surgical mortality in hyperthyroid cats (Birchard, Peterson & Jacobson, 1984). The most commonly used drug to treat cats prior to surgical thyroidectomy in the UK is the thioglyoxaline carbimazole. However, adverse effects (vomiting, anorexia and depression) requiring withdrawal of the drug, occur in approximately 8 per cent of treated cats (Mooney, Thoday & Doxey, 1992) and these cats usually go to surgery still thyrotoxic.

It has previously been shown in humans with hyperthyroidism caused by Graves' disease, that a combination of propranolol and potassium iodide (as a source of stable iodine) induced euthyroidism before surgical thyroidectomy (Feek, Sawers, Irvine *et al.*, 1980). A pharmaceutical preparation of potassium iodide is no longer available in the UK and potassium iodate is now being used in its place.

The aim of this prospective study was to determine whether a combination of propranolol and potassium iodate might be an effective method of preparing cats for surgical thyroidectomy.

8.02 MATERIALS AND METHODS

a) CLINICAL MATERIAL

The clinical material comprised a series of 23 hyperthyroid cats presented to the Small Animal Clinic of The University of Edinburgh's Royal (Dick) School of Veterinary Clinical Studies. A tentative diagnosis of hyperthyroidism was made on the basis of compatible historical and physical features and confirmed by the presence of elevated serum TT4 concentrations in all cases.

Before admission to the study, blood was collected by jugular venepuncture for haematological examinations and determination of a 13 parameter biochemical profile. Urine was collected by cystocentesis. Animals with identified concurrent non-thyroidal diseases were excluded from the study. After the informed consent of their owners, animals were assigned alternately to one of two groups (A and B) and hospitalised for the duration of the study.

b) STUDY PROTOCOLS

Group A cases received propranolol (Inderal, Zeneca) alone at 2.5mg/cat every eight hours from days 1 to 10 inclusive. The heart rate was determined each morning. If an animal's heart rate exceeded 200 beats/minute on day 4, the propranolol dosage was increased to 5mg/cat every eight hours and if it still exceeded this rate on day 7, to 7.5 mg/cat every 8 hours for the remainder of the 10 days. Subsequently, the same dose of propranolol and, initially, 42.5mg potassium iodate (equivalent to 25mg free iodine, Potassium Iodate tablets 85mg, Cambridge Self-Care Diagnostics) were administered every eight hours from days 11 to 20 until the time of surgery. The potassium iodate tablets were placed in gelatin capsules. Surgery was carried out on day 21. Potassium iodate was stopped immediately before thyroidectomy while propranolol was continued post-operatively for a further 3 days.

Group B cases were treated in the same way with the exception that they received potassium iodate alone from days 1 to 10 inclusive followed by potassium iodate and propranolol in combination from days 11 to 20 inclusive, with propranolol alone being continued post-operatively for a further three days.

Potassium iodate at the above dose appeared to cause an unacceptable degree of adverse effects (intermitent anorexia, vomiting and mild depression) in some cats. Consequently, the dosage was reduced to 21.25mg (equivalent to 12.5mg free iodine) and no further adverse effects were observed.

Cats were bled daily from day 1 to day 20 inclusive by jugular venepuncture. Two ml of whole blood was collected in a plain tube. Serum was harvested and stored at -40°C for subsequent assay of TT4, TT3 and rT3, which was carried out in respective batch modes. Complete blood counts were carried out at 5 day intervals on each cat to ensure that repeated blood sampling did not result in values outside the respective reference ranges. Serum potassium concentrations were determined in three cats which became anorexic on days 15 (one in group A and one in

group B, on 42.5 and 21.25mg potassium iodate respectively) and on day 17 in one cat in group A on 42.5mg potassium iodate.

c) LABORATORY STUDIES

Complete blood counts, serum biochemical tests and urine analyses were carried out by standard techniques.

Serum TT4 concentrations were determined using a double antibody RIA technique (DPC; TT4). Serum TT3 concentrations were determined using a single antibody RIA technique (Amerlex; MT3). Serum rT3 concentrations were determined using a single antibody RIA with PEG separation (Biostat Diagnostics; reverse T3).

The serum TT4 and TT3 assays were validated for use with cat serum by SCL Biosciences, Cambridge, U.K. The validation data is presented in Tables 8.01 and 8.02. The accuracy of the assays was assessed by determining the recovery of known added amounts of T4 or T3 which had been added to feline serum. For TT4 and TT3, the mean recovery of low and high concentrations in 12 experiments per analyte was 104.9 per cent and 97.9 per cent respectively. The specificity of the assays was demonstrated by dilutional parallelism. The precision of the assays and an acceptable degree of drift were assessed by the inclusion of low, medium and high pools of human serum (TT4 concentrations of 46, 103 and 201nmol/l respectively; TT3 concentrations of 0.82, 2.05 and 3.7nmol respectively) and one feline pool (TT4 concentration of 81.48nmol/l; TT3 concentration of 1.39nmol/l) in each assay. The mean within-assay coefficients of variation for TT4 and TT3 were 4 per cent and 8 per cent respectively and the mean between-assay coefficients of variation for TT4 and TT3 were 6.6 per cent and 6.0 per cent respectively. The working ranges of the assays were 4.0 to 300.0 nmol/l for TT4 (reference range 19.0 to 65.0 nmol/l) and 0.20 to 12.00 for TT3 (reference range 0.90 to 3.10 nmol/l).

The RIA for rT3 was not specifically optimised and validated for feline serum but each cat acted as its own control during the study. The cross reactivity of T4 and T3 in the Biodata radioimmuoassay kit for rT3 is approximately 0.029 and 0.0086 per cent respectively.

d) STATISTICAL ANALYSES

The Friedman test was used to examine overall differences in thyroid hormone concentrations between days 1 to 20 within the treatment groups. The Wilcoxon Signed Rank test was used to test for differences in heart rates and thyroid hormone concentrations between days 1, 11 and 20 within groups. The Mann-Whitney U test was used to examine differences in heart rates and thyroid hormone concentrations between days 1, 11 and 20 between groups. With all tests, a p value of < 0.05 was considered significant.

Table 8.01: Recovery data for total L-thyroxine (T4) and total L-triiodothyronine (TT3) assays

Expected result	Actual value	Per cent recovery
TT4(nmol/l)		
Recovery pool 1		
48	49	102.1
48	47	97.9
48	49	102.1
65	74	113.8
65	72	110.8
65	70	107.7
Recovery pool 2		
65	66	101.5
65	64	98.5
65	67	103.1
82	91	111.0
82	86	104.9
82	86	104.9
	Mean	**104.9**
TT3 (nmol/l)		
Recovery pool 1		
0.73	0.68	93.2
0.73	0.65	89.0
0.73	0.66	90.4
1.75	1.82	104.0
1.75	1.82	104.0
1.75	1.83	104.6
Recovery pool 2		
1.43	1.35	94.4
1.43	1.34	93.7
1.43	1.34	93.7
2.45	2.54	103.7
2.45	2.50	102.0
2.45	2.50	102.0
	Mean	**97.9**

Table 8.02: Dilutional parallelism data for the total L-thyroxine (TT4) and total L-triiodothyronine (TT3) assays.

Dilution	Expected	Actual	Per cent deviation
TT4 (nmol/l)			
1:1	191	191	0
1:2	96	92	-4.2
1:4	48	46	-4.2
1:8	24	21	-12.5
1:16	11.9	10	-16.0
1:32	5.9	5.3	-10.2
TT3 (nmol/l)			
1:1	7.85	7.85	0
1:2	3.93	3.71	-5.6
1:4	1.96	1.76	-10.2
1:8	0.98	0.79	-19.4
1:16	0.49	0.40	-18.4
1:32	0.24	0.19	-20.8

8.03 RESULTS

Twenty-three cats were admitted to the trial. One cat had to be withdrawn from group A on day 8 due to the development of congestive cardiac failure and was successfully treated with carbimazole followed by surgical thyroidectomy. One cat in Group A died suddenly on day 8 of treatment due to an hepatic infarct.

Twenty-one cats completed the trial with 11 in Group A and 10 in Group B (Table 8.03), although one cat in each group died on induction of anaesthesia. There were 16 Domestic Shorthaired and five Domestic Longhaired animals with 15 being ovariohysterectomised females and six castrated males (three castrated males being present in each of the groups). The median age was 13 years (range 7 to 16 years). There was no significant difference in the age of the cats between groups A (median 13 years; range 7 to 16 years) and B (median 12.5 years, range 9 to 15 years) ($p = 0.8603$).

a) GROUP A CATS

In this group, four cats were treated with 42.5mg three times daily and seven were treated with 21.25mg of potassium iodate three times daily. The only cat in the study which required 7.5mg propranolol three times daily was in this group.

i) Clinical Response

The drugs were reasonably well-tolerated by most cats. No adverse effects were noted when the cats were taking the propranolol alone. However, intermittent anorexia and mild depression were seen in six of the 11 cats in this group once potassium iodate was introduced. Of the six cats that developed inappetance, three were on 42.5 mg three times daily and three were on 21.25 mg of potassium iodate three times daily. Six cats vomited at some point during the trial, two of which which had vomiting as part of their previous history, with four cats vomiting whilst on propranolol alone.

Figure 8.01 shows the heart rates of the cats in group A over the study period and the data are summarised in Table 8.04.

Heart rates were well-controlled by the propranolol alone and fell significantly from days 1 to 11 ($p = 0.0040$), over the entire treatment period (days 1 to 20) ($p = 0.0040$) but not from days 11 to 20 ($p = 0.2490$).

ii) Thyroid Hormone Concentrations

Figure 8.02 shows the serum TT4 concentrations of the cats in group A over the study period and the data are summarised in Table 8.05.

Of the 11 cats in this group, all had elevated TT4 concentrations on day 1 of the study. Of these, four (36 per cent) had normal TT4 concentrations on day 20 of the study. The serum TT4 concentrations on day 20 were significantly lower than those on day 1 ($p = 0.0370$) and there was a significant difference between the days over the treatment period ($p = 0.0000$). There was no significant difference in TT4 concentrations between the first and last days of propranolol treatment alone (day 1 compared to day 11) ($p = 0.3070$) but the TT4 concentrations fell significantly between the first and last days of combined propranolol and potassium iodate treatment (day 11 compared to day 20) ($p = 0.0370$).

Figure 8.03 shows the serum TT3 concentrations of the cats in group A over the study period and the data are summarised in Table 8.05.

Of the 11 cats, nine (82 per cent) had elevated total T3 concentrations at the time of diagnosis and eight of these (89 per cent) had reference range TT3 concentrations at the time of surgery. The serum TT3 concentrations on day 20 were significantly lower than those on day 1 ($p = 0.0040$), and there was a significant difference between the days over the treatment period ($p = 0.000$). There was a significant fall in the serum TT3 concentrations between day 1 and day 11 ($p = 0.0370$) and between days 11 and 20 ($p = 0.0040$).

Figure 8.04 shows the serum rT3 concentrations of the cats in group A over the study period and the data are summarised in Table 8.05.

The serum rT3 concentrations on day 20 were significantly lower than on day 1 ($p = 0.0040$). The rT3 concentrations did not change significantly from days 1 to 11 ($p = 0.1850$) but fell significantly from days 11 to 20 ($p = 0.0090$).

b) GROUP B CATS

In this group, four cats were treated with 42.5 mg potassium iodate three times daily and sixwere treated with 21.25 mg of potassium iodate three times daily.

i) Clinical Response

This regimen seemed less well-tolerated by the cats generally. Anorexia and depression were noted in four cats (three on 42.5 mg potassium iodate three times daily and one on 21.25 mg potassium iodate three times daily). Jaundice developed in two cats being given 42.5 mg potassium iodate towards the end of the treatment period (days 16 and 18 respectively). Both completed the study. One underwent surgical thyroidectomy and liver biopsy via exploratory laparotomy. Histopathological examination revealed hepatic lipidosis. The cat survived for 7 days but subsequently died due to complications of its liver disease. A further cat in this group also died on induction of anaesthesia. Post-mortem examination revealed no abnormalities in this cat other than bilateral thyroid adenomatous hyperplasia. Eight out of 10 cats vomited during the trial, five of which were presented for vomiting as part of their previous history.

Figure 8.05 shows the heart rates of the cats in group B over the study period and the data are summarised in Table 8.04.

Heart rates were best controlled when propranolol therapy had begun but fell significantly from days 1 to 20 ($p = 0.0060$), from days 1 to 11 ($p = 0.0060$) and from days 11 to 20 ($p = 0.0210$).

ii) Thyroid Hormone Concentrations

Figure 8.06 shows the serum TT4 concentrations of the 10 cats in group B over the study period and the data are summarised in Table 8.05.

Of the 10 cats in this group, all had elevated serum TT4 concentrations on day 1 of the study. Of these, one (10 per cent) had a normal serum TT4 concentration on day 20 of the study, although a further three cats were transiently euthyroid (during days 4 to 15 inclusive [two cats] and days 16 and 17 [one]). The serum TT4 concentrations on day 20 were not significantly lower than those on day 1 ($p = 0.2340$), although there was a significant difference between the days over the treatment period ($p = 0.0020$). This may have been due to the outlier on day 20. When this cat's data were removed, the serum TT4 concentrations on day 20 were significantly lower than those on day 1 ($p = 0.0350$). TT4 concentrations fell significantly between the first and last days of potassium iodate treatment alone (day 1 compared to day 11) ($p = 0.041$), but not between the

first and last days of combined propranolol and potassium iodate treatment (day 11 compared to day 20) ($p = 0.2800$). The removal of the outlier in this case did not affect this result ($p = 0.4470$).

Figure 8.07 shows the serum TT3 concentrations of the cats in group B over the study period and the data are summarised in Table 8.05.

Of the 10 cats in group B, four (40 per cent) had elevated TT3 concentrations on day 1 of the study. Of these four cats, three (75 per cent) had normal TT3 concentrations at the time of surgery. The serum TT3 concentrations on day 20 were not significantly lower than those on day 1 ($p = 0.1240$), although there was a significant difference between days over the treatment period ($p = 0.002$). There was no significant fall in serum TT3 concentrations from days 1 to 11 ($p = 0.1390$) or from days 11 to 20 ($p = 0.7220$). The removal of the outlying data did not affect the statistical outcome.

Figure 8.08 shows the serum rT3 concentrations of the cats in group B over the study period and the data are summarised in Table 8.05.

The serum rT3 concentrations were not significantly different between days 1 and 20 ($p = 0.6360$), between days 1 and 11 ($p = 0.4760$) or 11 and 20 ($p = 0.2860$). The removal of the outlying data did not affect the statistical outcome.

c) COMPLETE BLOOD COUNTS

No significant abnormalities attributable to repeated blood sampling were seen in any parameter in any cat in the study.

d) SERUM POTASSIUM CONCENTRATIONS

The three anorexic cats which had serum potassium concentrations determined all showed values within the reference range (4.00 to 5.00 nmol/l).

e) COMPARISON OF GROUP A AND GROUP B CATS

i) Heart Rates

The data comparing the heart rates of the cats in Groups A and B are summarised in Table 8.02.

There was no significant difference in heart rates between group A and B cats at days 1, 11 or 20 ($p = 0.9439$, 0.1809 and 0.3981 respectively).

ii) Thyroid Hormone Concentrations

The data comparing the serum TT4, TT3 and rT3 concentrations of the cats in Groups A and B are summarised in Table 8.03.

For serum TT4 concentrations, there was no significant difference between group A and B cats at days 1, 11 or 20 ($p = 0.3418$, 0.6472 and 0.3069 respectively). Scatter plots showing data points for serum TT4 concentrations of individual cats in Groups A and B on day 1 and day 20 are shown in Figure 8.09.

For serum TT3 concentrations, there was no significant differences between group A and B cats at days 1, 11 or 20 ($p =$ 0.5035, 0.1300 and 0.3823 respectively). Scatter plots showing data points for serum TT3 concentrations of individual cats in Groups A and B on day 1 and day 20 are shown in Figure 8.10.

For serum rT3 concentrations, there was no significant difference between group A and B cats at days 1, 11 or 20 ($p = 1.000$, 0.3847, and 0.4033 respectively). Scatter plots showing data points for serum rT3 concentrations of individual cats in Groups A and B on day 1 and day 20 are shown in Figure 8.11.

f) LIVER PATHOLOGY

Histological examination of the liver was carried out on three cats, identified by the numbers in Table 1. All were taking 42.5mg of potassium iodate three times daily. Cat 3 was in Group A and was the sole cat taking 7.5 mg propranolol three times daily from day 7 of the study. This cat died on anaesthetic induction. Liver pathology showed hepatocellular necrosis, with minimal periportal lymphocyte and neutrophil infiltration, indicative of mild cholangitis. The liver changes were not considered severe enough to have contributed to the cat's death.

Two (cats 14 and 15) were in Group B and were taking 5mg propranolol three times daily when they developed jaundice. Cat 14 was improving at the end of the trial when it had an ultrasound-guided needle liver biospy. Liver histology from this cat showed mild fatty degeneration together with acute cholangitis. Cat 15 had a liver biopsy at the time of thyroidectomy. The biopsy results showed focal fatty nodules and areas of fatty degeneration of cytoplasm indicative of hepatic lipidosis. However, at post-mortem examination, histological examination showed more chronic degenerative changes without significant associated fatty abnormalities.

Table 8.03:Summary Of Clinical Details Of The Cats Which Completed The Study

GROUP A CATS

CAT	AGE (years)	BREED	SEX	REASON FOR PRESENTATION	DOSE OF POTASSIUM IODATE (TID) DURING TRIAL (mg)	TT4 REFERENCE RANGE AT ANY TIME DURING TRIAL	TT4 REFERENCE RANGE AT TIME OF SURGERY
1	13	DSH	MN	Vaccination	42.50	No	No
2	14	DLH	FN	Vomiting	42.50	No	No
3	15	DSH	MN	Weight loss	42.50	No	No
4	14	DSH	FN	Weight loss	42.50	Yes	Yes
5	16	DSH	FN	Vaccination	21.25	No	No
6	9	DLH	FN	Weight loss	21.25	Yes	No
7	7	DSH	FN	Weight loss	21.25	No	No
8	8	DSH	FN	Polyphagia	21.25	No	No
9	8	DSH	FN	Heart murmur	21.25	Yes	Yes
10	12	DSH	MN	Weight loss	21.25	Yes	Yes
11	15	DSH	FN	Weight loss	21.25	Yes	Yes

GROUP B CATS

CAT	AGE (years)	BREED	SEX	REASON FOR PRESENTATION	DOSE OF POTASSIUM IODATE (TID) DURING TRIAL (mg)	TT4 REFERENCE RANGE AT ANY TIME DURING TRIAL	TT4 REFERENCE RANGE AT TIME OF SURGERY
12	15	DLH	MN	Vomiting	42.50	No	No
13	13	DSH	FN	PU/PD	42.50	No	No
14	10	DSH	FN	Weight loss	42.50	No	No
15	14	DSH	MN	Weight loss	42.50	Yes	No
16	14	DSH	FN	Polyphagia	21.25	No	No
17	10	DLH	FN	Weight loss	21.25	Yes	Yes
18	9	DSH	FN	Weight loss	21.25	Yes	No
19	15	DLH	MN	Vaccination	21.25	No	No
20	12	DSH	FN	Matted fur	21.25	No	No
21	12	DSH	FN	Anorexia	21.25	Yes	No

DLH, Domestic Long Hair; DSH, Domestic Short Hair; FN, Ovariohysterectomised female; MN, Castrated male; PD, Polydipsia; PU, Polyuria; TID, Three times daily, TT4, Total serum thyroxine concentration

Table 8.04: Comparison Of Heart Rates Of Cats In Groups A and B Before, During And At The End Of Treatment Periods With Propranolol And Potassium Iodate

		Heart Rate (beats per minute)		
Cat Group	Day	Range	Median	Quartiles
A	1	180 - 280	240	220 260
	11	140 - 220	180	178 200
	20	140 - 200	180	160 180
B	1	180 - 270	250	195 260
	11	140 - 240	200	170 220
	20	140 - 210	180	175 200

Group A cats had propranolol from days 1 to 20 and potassium iodate from days 11 to 20 inclusive. Group B cats had potassium iodate from days 1 to 20 and propranolol from days 11 to 20 inclusive

Table 8.05: Comparison Of Serum Thyroid Hormone Concentrations Of Cats In Groups A And B Before, During And At the End Of The Treatment Periods With Propranolol And Potassium Iodate.

			Thyroid Hormone Concentration (nmol/l)		
Thyroid Hormone	Cat Group	Day	Range	Median	Quartiles
Thyroxine	A	1	88.8 - 275.6	165.6	104.4 214.6
		11	62.5 - 275.8	155.7	95.4 173.2
		20	45.3 - 264.1	97.5	46.2 120.1
	B	1	88.6 - 313.2	219.7	105.7 285.3
		11	39.2 - 279.5	120.1	26.3 220.4
		20	39.5 - 453.5	120.1	84.0 212.9
Triiodothyronine	A	1	3.16 - 5.26	4.06	3.48 4.37
		11	2.47 - 4.74	2.84	2.68 3.49
		20	1.18 - 2.60	1.76	1.36 2.38
	B	1	1.95 - 10.63	2.94	2.19 4.61
		11	0.54 - 5.87	1.91	0.83 3.05
		20	1.00 - 7.85	1.95	1.63 2.31
reverse Triiodothyronine	A	1	0.75 - 9.30	3.70	1.22 4.95
		11	0.62 - 5.55	3.05	1.21 4.36
		20	0.50 - 3.30	1.69	0.72 2.10
	B	1	0.87 - 9.50	2.64	1.21 8.43
		11	0.54 - 4.90	1.72	0.86 3.88
		20	0.31 - 7.45	2.60	1.07 3.13

Group A cats had propranolol from days 1 to 20 and potassium iodate from days 11 to 20 inclusive. Group B cats had potassium iodate from days 1 to 20 and propranolol from days 11 to 20 inclusive

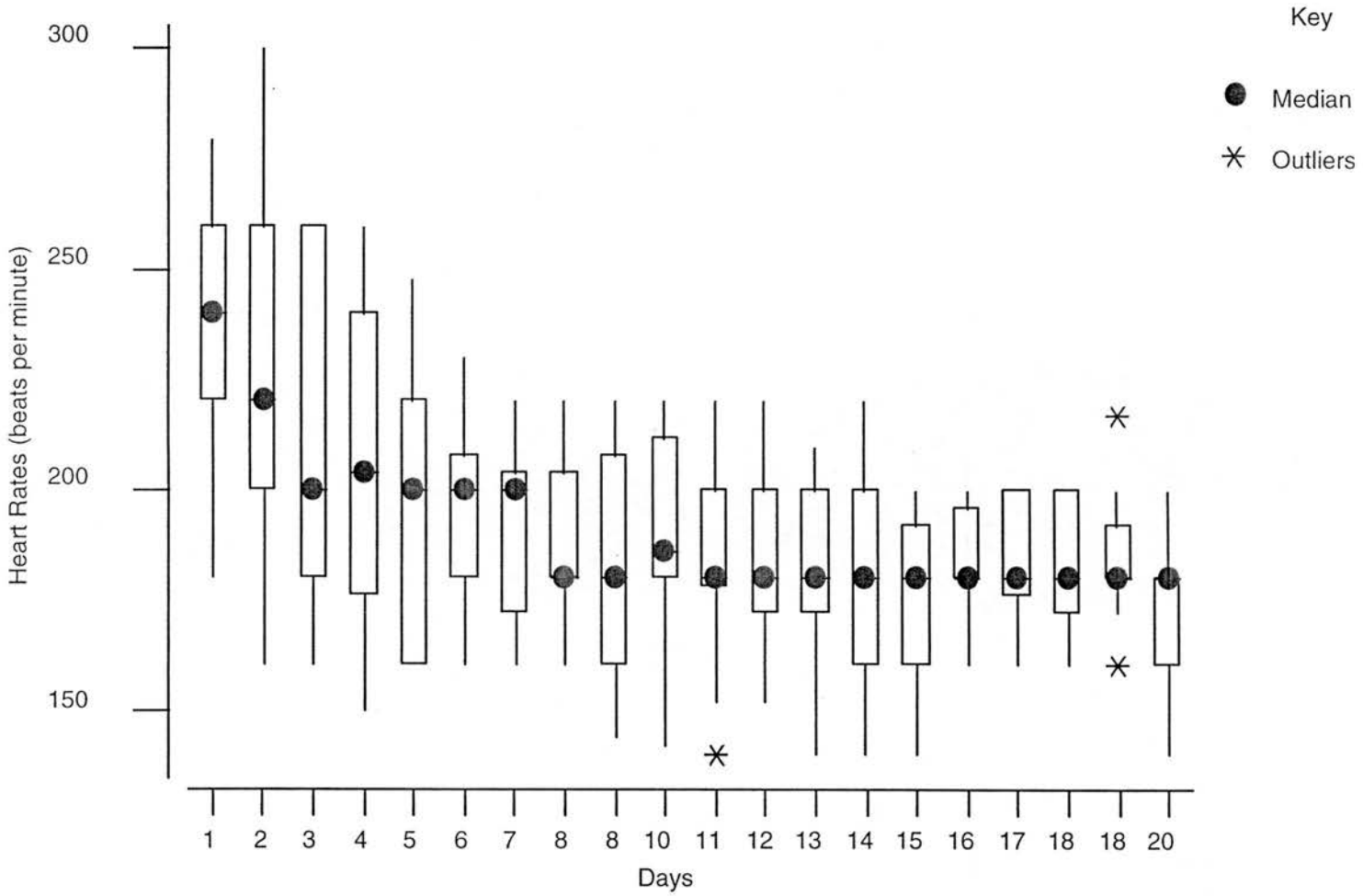


Figure 8.01: Boxplot of Group A cats showing heart rates over the 20 day trial period. Group A cats received propranolol for the first 10 days and a combination of propranolol and potassium iodate for the following 10 days. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3). Whiskers define the range of the data.

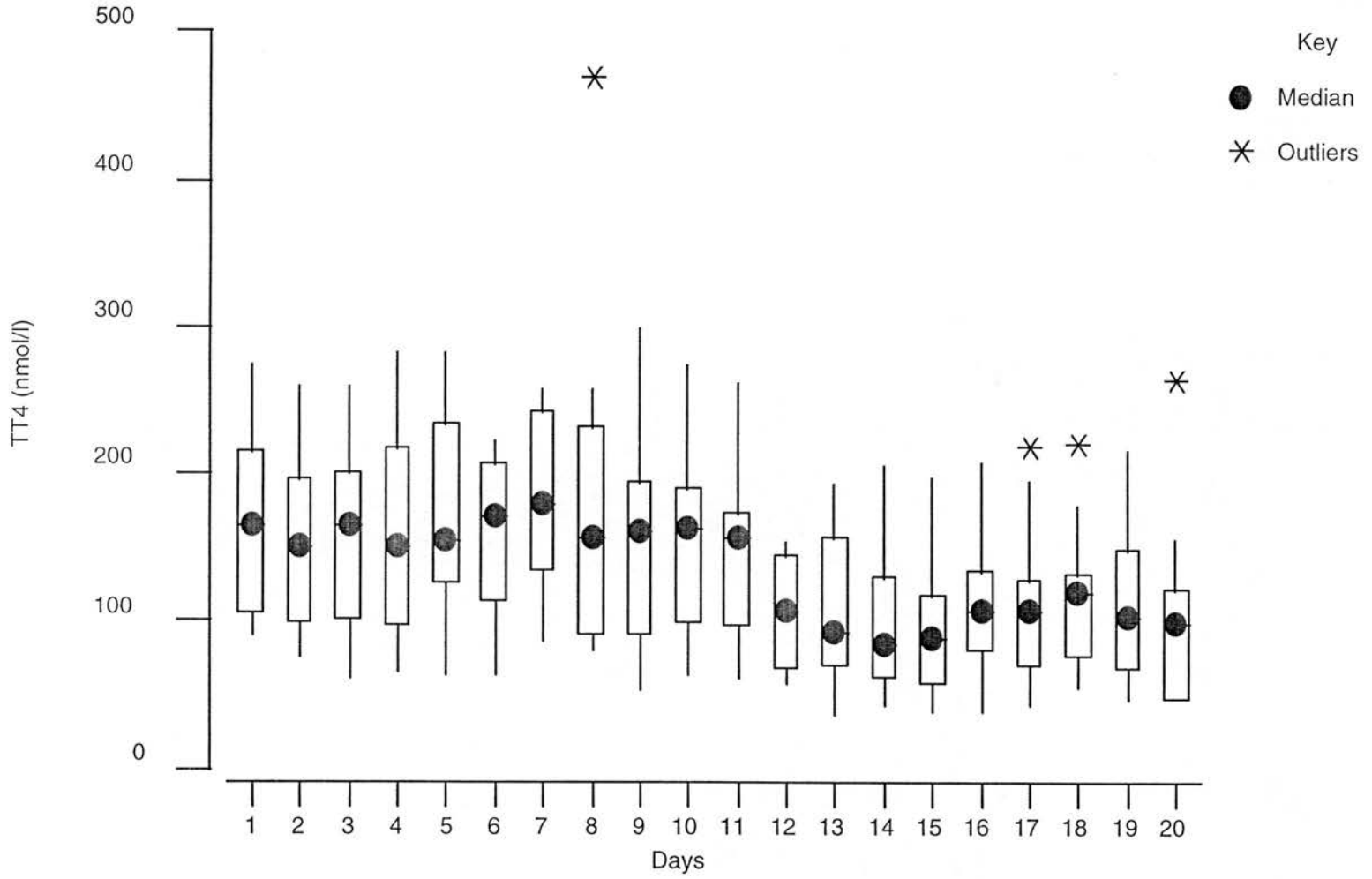


Figure 8.02: Boxplot of Group A cats showing serum total thyroxine (TT4) concentrations over the 20 day trial period. Group A cats received propranolol for the first 10 days and a combination of propranolol and potassium iodate for the following 10 days. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3). Whiskers define the range of the data.

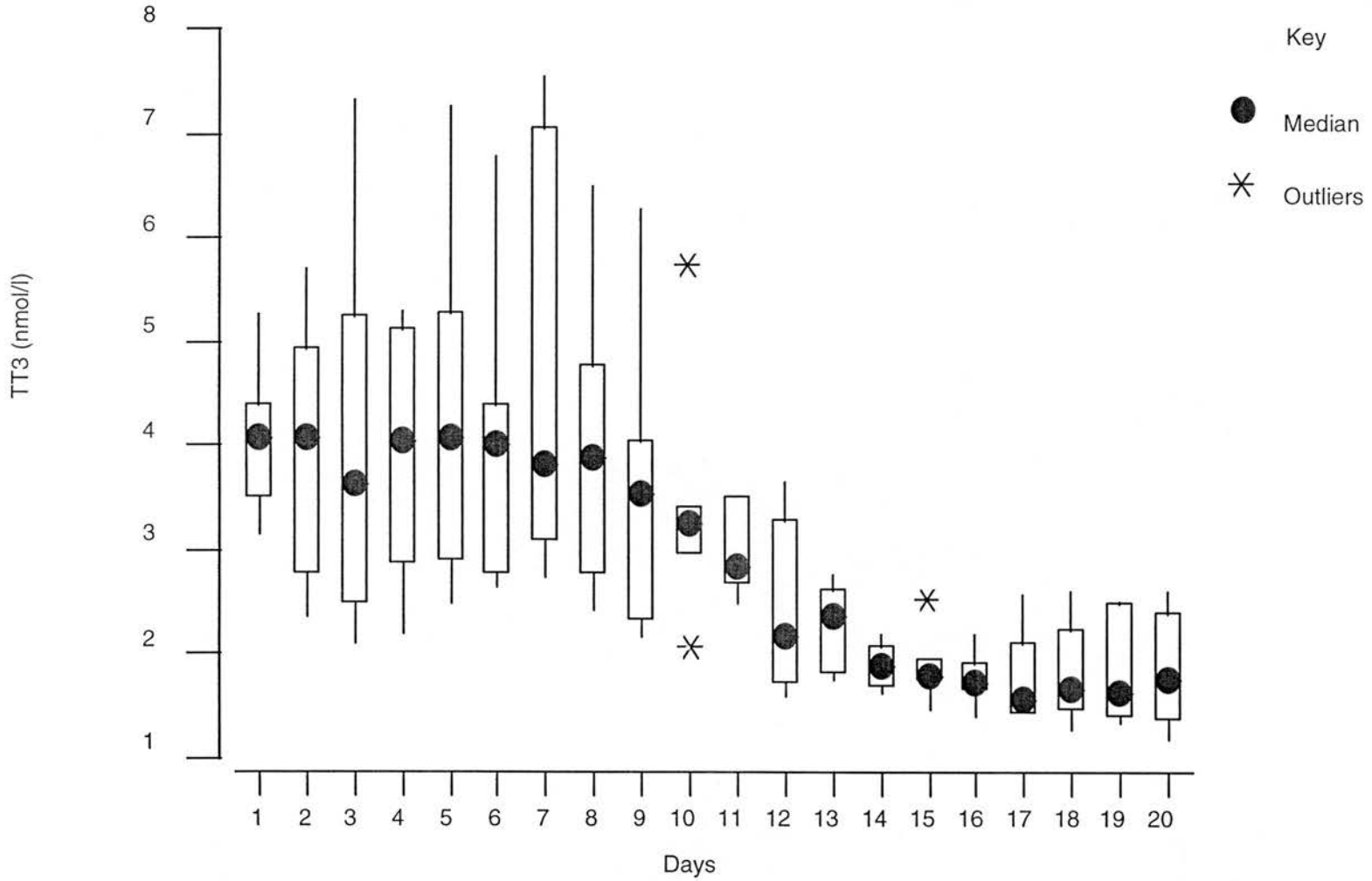


Figure 8.03: Boxplot of Group A cats showing total triiodothyronine (TT3) concentrations over the 20 day trial period. Group A cats received propranolol for the first 10 days and a combination of propranolol and potassium iodate for the following 10 days. Bottom of the box represents the first quartile (Q1), the top of the box the third quartile (Q3). Whiskers define the range of the data.

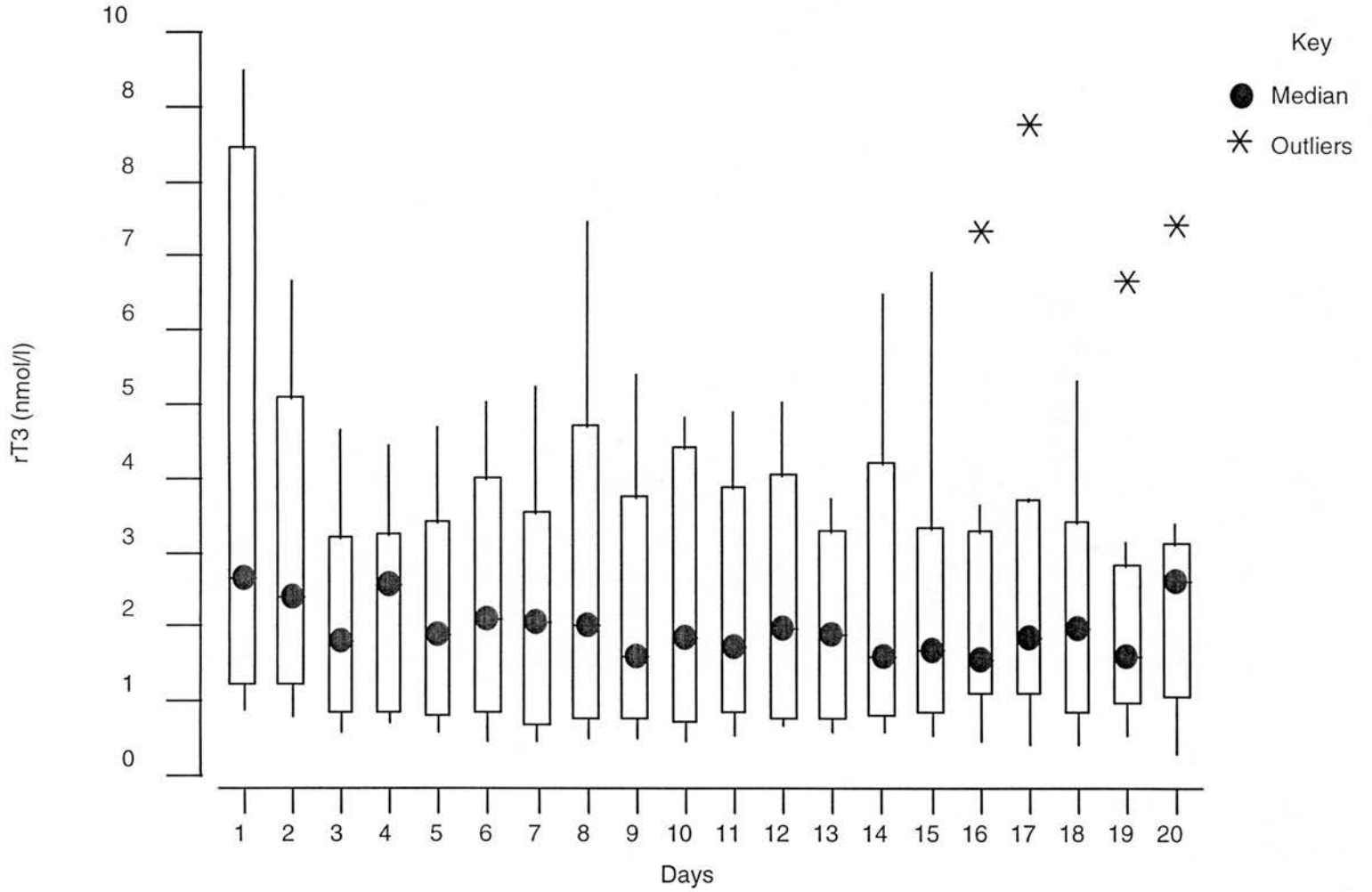


Figure 8.04: Boxplot of Group A cats showing rT3 concentrations over the 20 day trial period. Group A cats received propranolol for the first 10 days and a combination of propranolol and potassium iodate for the following 10 days. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3). Whiskers define the range of the data.

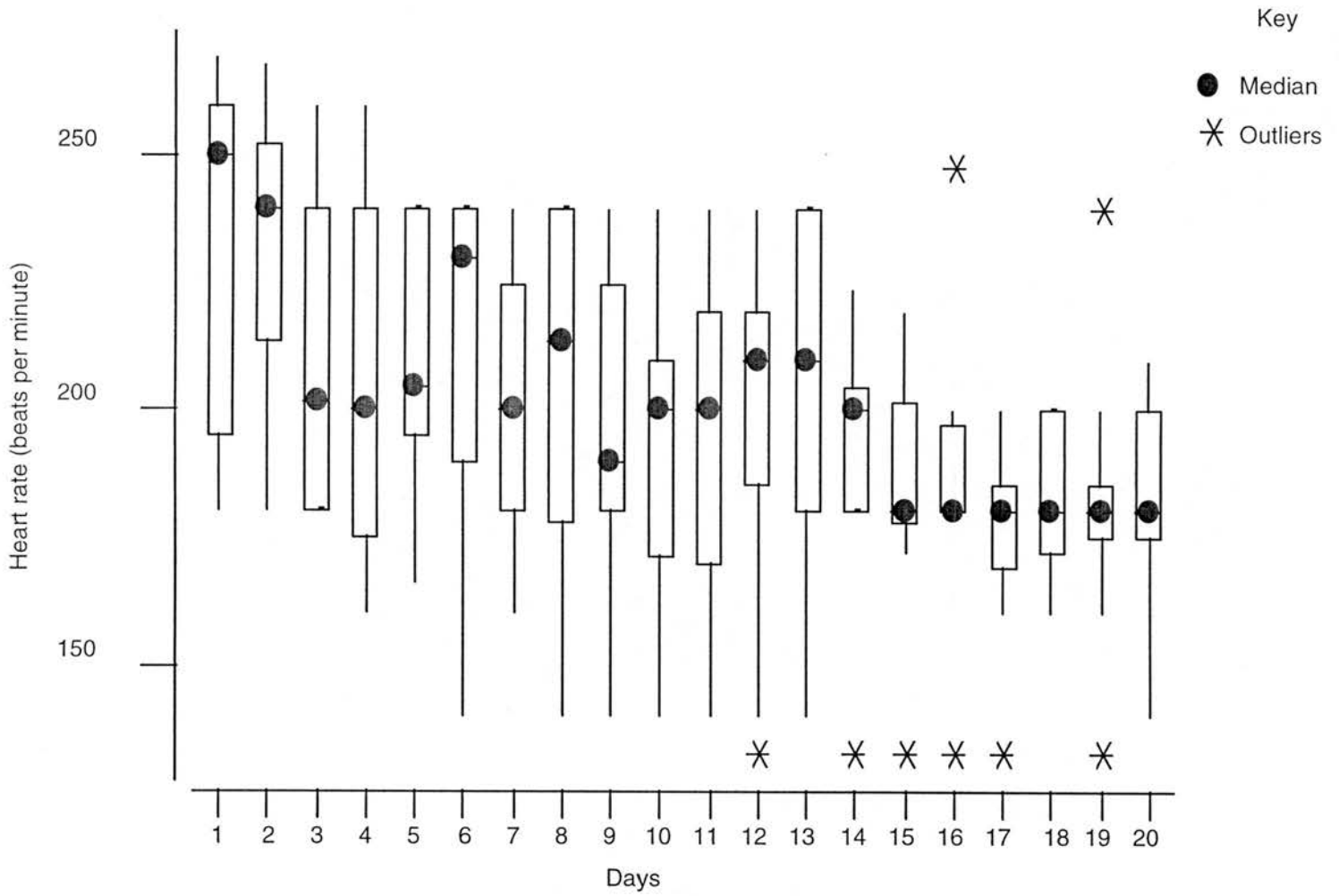


Figure 8.05: Boxplot of Group B cats showing heart rates over the 20 day trial period. Group B cats received potassium iodate for the first 10 days and a combination of potassium iodate and propranolol for the following 10 days. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3.Whiskers define the range of the data.

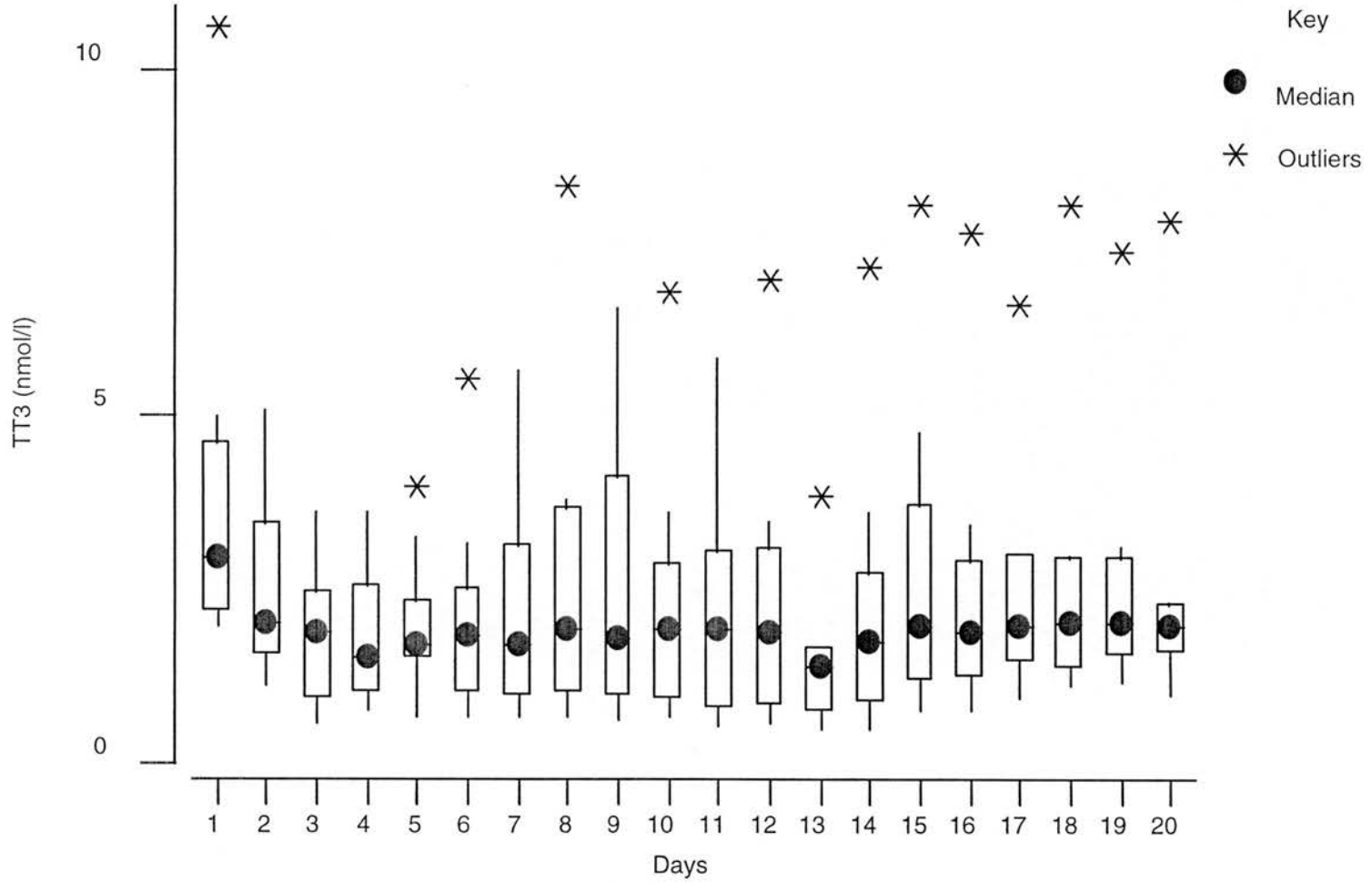


Figure 8.07: Boxplot of Group B cats showing serum total T3 concentrations over the 20 day trial period. Group B cats received potassium iodate for the first 10 days and a combination of potassium iodate and propranolol for the following 10 days. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3). Whiskers define the range of the data.

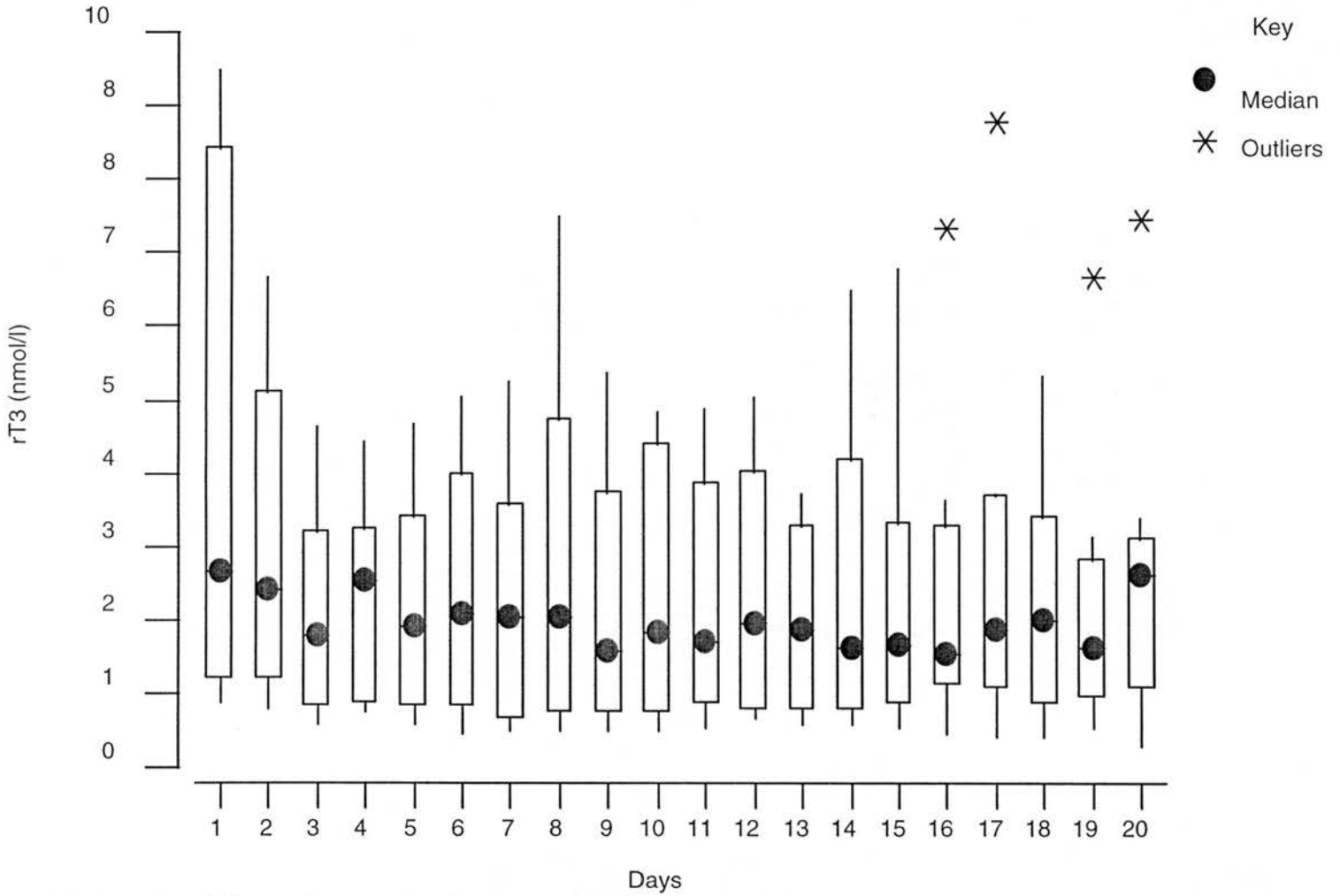


Figure 8.08: Boxplot of Group B cats showing serum total reverse T3 (rT3) concentrations over the 20 day trial period. Group B cats received potassium iodate for the first 10 days and a combination of potassium iodate and propranolol for the following 10 days. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3). Whiskers define the range of the data.

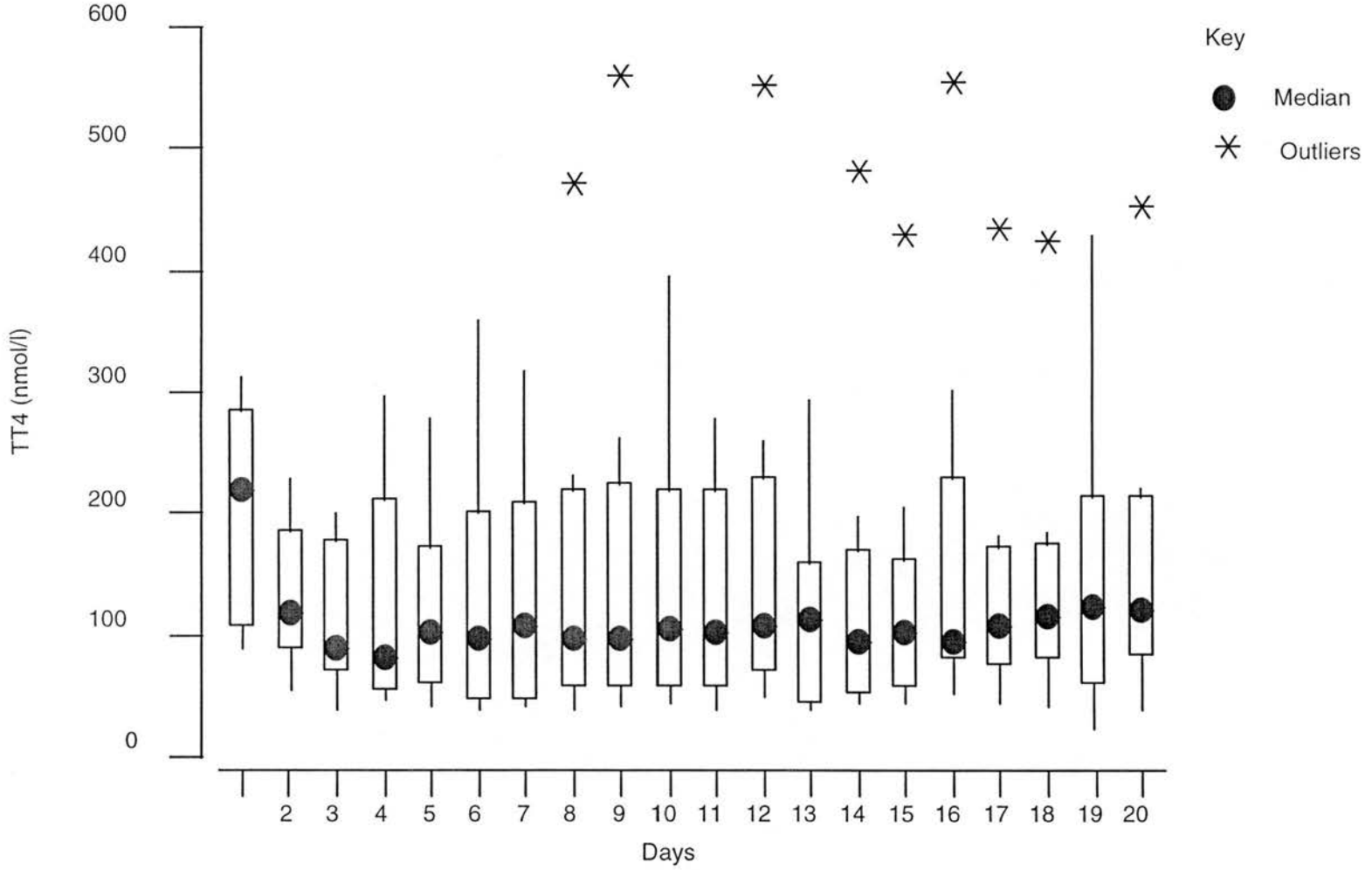


Figure 8.06: Boxplot of Group B cats showing serum total T4 concentrations over the 20 day trial period. Group B cats received potassium iodate for the first 10 days and a combination of potassium iodate and propranolol for the following 10 days. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3). Whiskers define the range of the data.

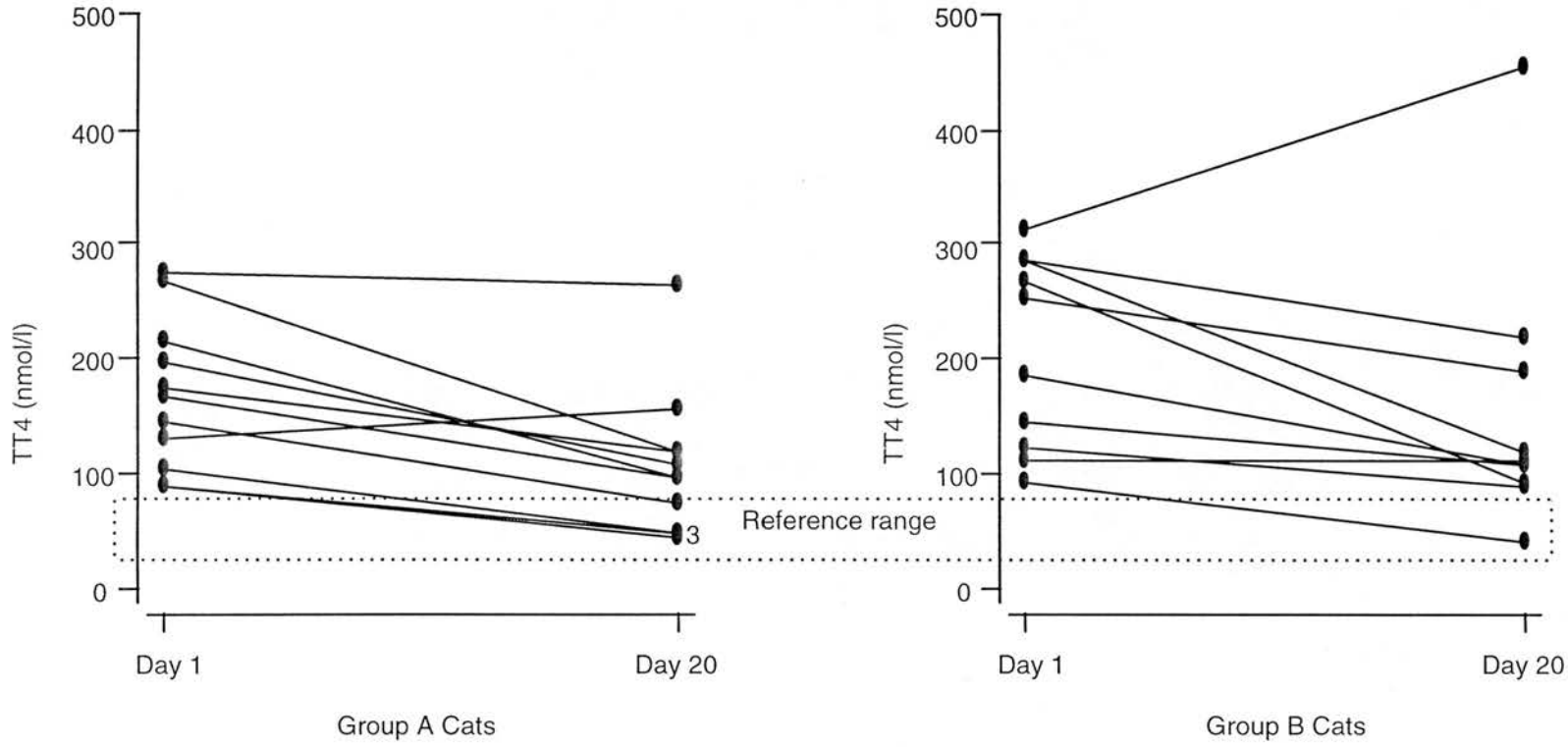


Figure 8.08: Scatter Plots of Group A and B cats showing serum total T4 (TT4) concentrations on days 1 and 20. Group A cats received propranolol for the first 10 days and a combination of propranolol and potassium iodate for the following 10 days. Group B cats received potassium iodate for the first 10 days and a combination of potassium iodate and propranolol for the following 10 days. The reference range for serum TT4 concentrations (18 to 65nmol/l) is illustrated by the boxed region.

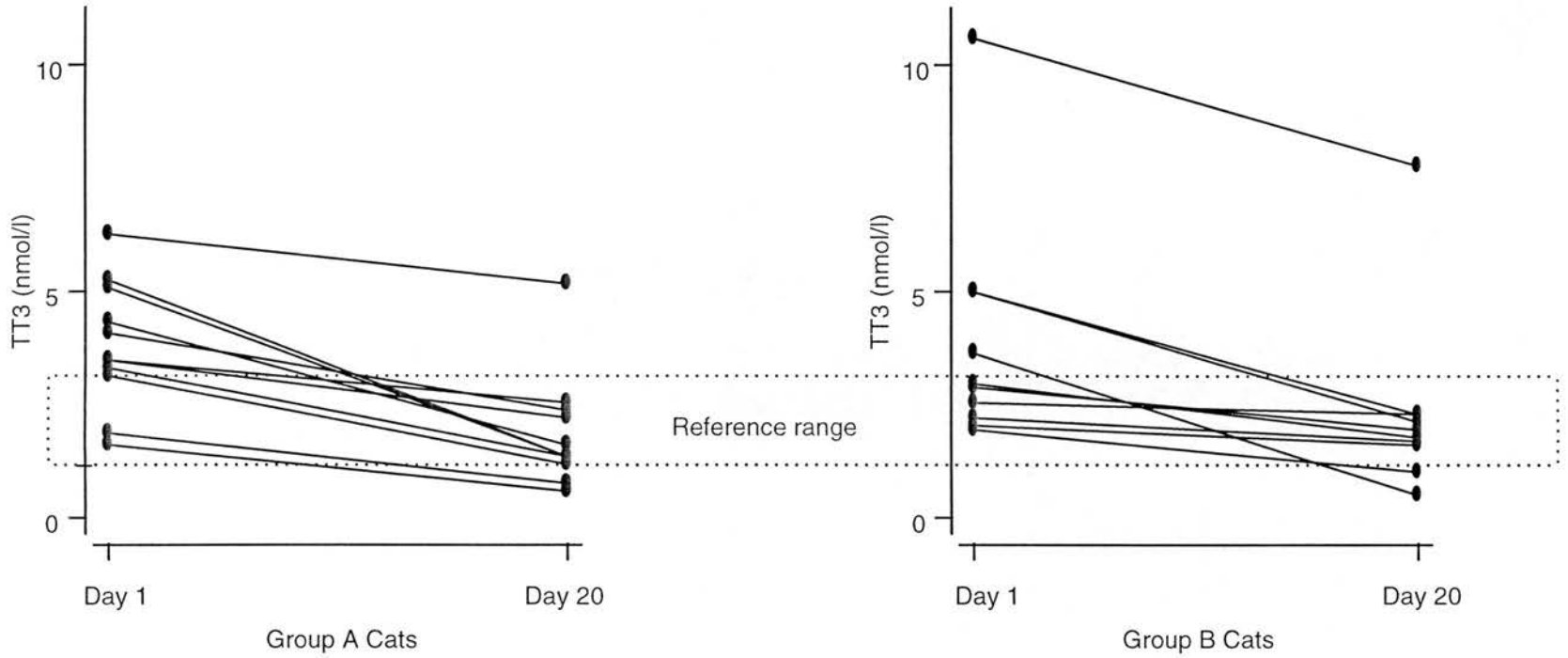


Figure 8.10: Scatter Plots of Group A and B cats showing serum total T3 (TT3) concentrations on days 1 and 20. Group A cats received propranolol for the first 10 days and a combination of propranolol and potassium iodate for the following 10 days. Group B cats received potassium iodate for the first 10 days and a combination of potassium iodate and propranolol for the following 10 days. The reference range for serum TT3 concentrations (0.8 to 3.1nmol/l) is illustrated by the boxed region.

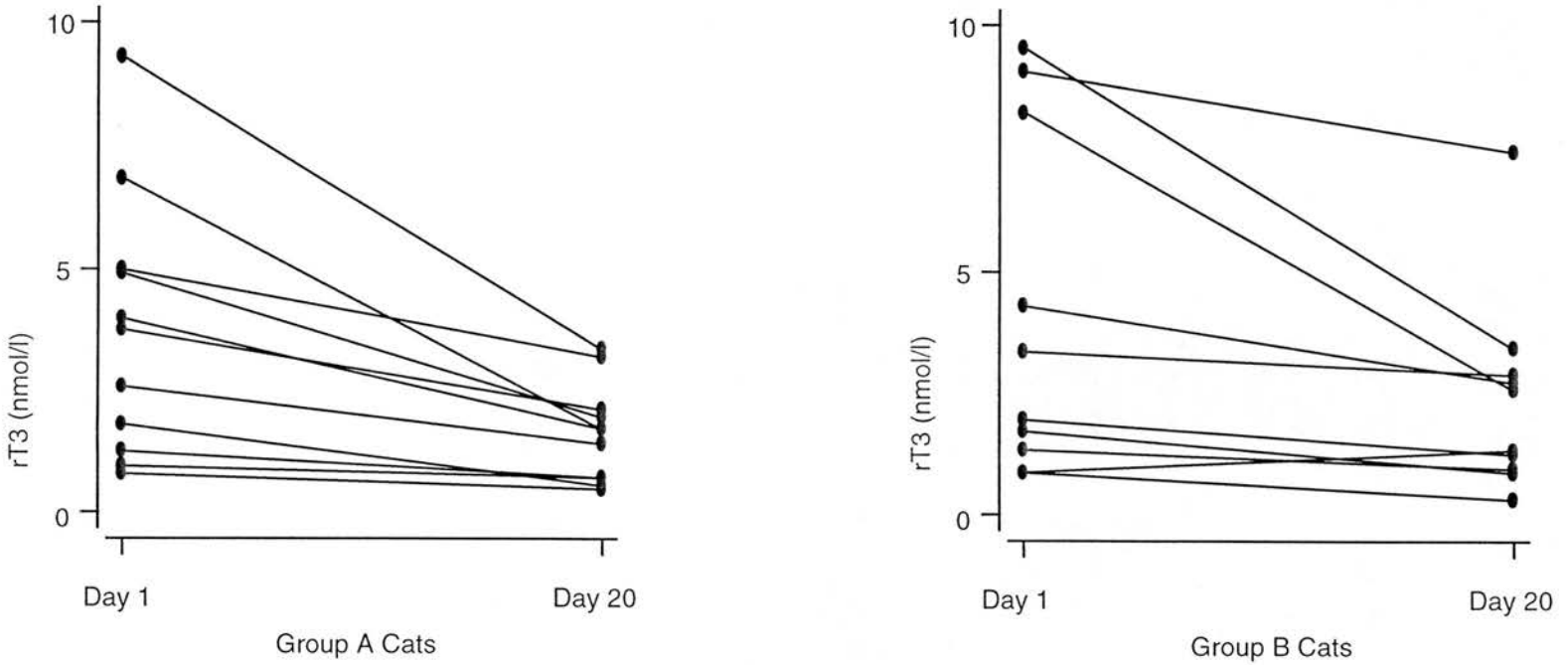


Figure 8.11: Scatter Plots of Group A and B cats showing serum total reverse T3 (rT3) concentrations on days 1 and 20. Group A cats received propranolol for the first 10 days and a combination of propranolol and potassium iodate for the following 10 days. Group B cats received potassium iodate for the first 10 days and a combination of potassium iodate and propranolol for the following 10 days.

8.04 DISCUSSION

Feline hyperthyroidism is the most commonly diagnosed endocrine disease of cats (Thoday and Mooney, 1992a). Despite a number of treatment options, the most reliable in the medium to long-term for the practitioner is surgical thyroidectomy. As hyperthyroid cats are poor anaesthetic risks (Peterson and Becker, 1983) it is recommended that euthyroidism is induced prior to surgery (Birchard, Peterson and Jacobson, 1984). The drugs of choice are the thioglyoxalines carbimazole (NeoMercazole, Roche) available in Europe and methimazole (Tapazole, Eli Lilly) available in the U.S.A.

Mild clinical adverse reactions (anorexia, vomiting and lethargy) requiring drug withdrawal occur in approximately 8 per cent and 15 per cent of cats treated with carbimazole and methimazole respectively (Peterson, Kintzer and Hurvitz, 1988; Mooney, Thoday and Doxey, 1992). Serious adverse effects, such as thrombocytopenia and agranulocytosis, have been reported in up to 3 per cent of cats treated with methimazole (Peterson, Kintzer and Hurvitz, 1988) but not in cats treated with carbimazole.

It is clear, therefore, that an alternative method of preparation for surgery is required for a relatively small percentage, but, because of the high incidence of the disease, a very significant number, of hyperthyroid cats.

In this study, two groups were chosen to examine initially the independent action of propranolol and potassium iodate and then to investigate their combined effect. As thyroid hormone concentrations may fluctuate markedly in hyperthyroid cats not receiving any form of therapy (Peterson, Graves and Cavanagh, 1987) and, in some cats, TT3 concentrations may fall rapidly when treated with potassium iodate and propranolol (Thoday, 1986), blood sampling was carried out daily. No adverse effects on haematological parameters from this protocol were noted.

The cases included in the trial comprised a combination of Domestic Short and Longhaired cats, with a median age in the two groups of 12.5 and 13 years. This part of the signalment is highly comparable to previously reported series of hyperthyroid cats (Peterson, Kintzer, Cavanagh *et al.*, 1983; Thoday and Mooney, 1992a). There were 2.5 times the number of ovariohysterectomised females than castrated males. However, this is likely to have arisen by chance as there is no reported sex predisposition for feline hyperthyroidism, either in our clinic (Thoday and Mooney, 1992a) or elsewhere (Hoenig, Goldschmidt, Ferguson *et al.*, 1982; Peterson, Kintzer, Cavanagh *et al.*, 1983). As the study was designed to evaluate the effect of

the drugs on serum thyroid hormone concentrations and as concurrent diseases may affect these, animals with any identified non-thyroidal diseases were excluded from the study.

In the human Graves' disease study (Feek, Sawers, Irvine *et al.*, 1980), patients were treated with 60mg potassium iodide (yielding 33mg free iodine) three times daily. A proportionately higher dose of free iodine (25mg three times daily) was initially administered to the cats in this study to ensure that any potential effect would be induced. Although this dose was subsequently reduced to 12.5mg three times daily, this is unlikely to have had any effect on the outcome of the study as 3.3mg free iodine three times daily has previously been shown to have significant inhibitory effects on thyroid hormone production in cats (Thoday, 1986). The dose of propranolol used (2.5 to 7.5 mg per cat three times daily) has been shown to be effective in blocking the increased sympathetic effects of hyperthyroidism in most affected cats. Efficacy was determined by reduction of heart rate to below 200 beats per minute. This has been reported to be an appropriate clinical method of determining adequate ß-adrenergic blockade in cats (Jacobs, Whitten, Sama *et al.*, 1997).

At the beginning of the trial, all cats had elevated TT4 concentrations, while a number in both groups had reference range TT3 concentrations. The latter has been well-recorded in hyperthyroid cats and may result from within- or between-day variations in mildly affected animals or the effects of non-thyroidal illness (Peterson, Graves and Cavanagh, 1987; Peterson and Gamble, 1988). As cats with non-thyroidal illness were, as far as could be determined, excluded from the study, the reference range TT3 values in some cats at the beginning of this study probably resulted from intra- or inter-daily variations which is the far commoner cause in feline thyrotoxicosis (Thoday and Mooney, 1992a). No cat that had a TT4 concentration at diagnosis of more than 190nmol/l attained a reference range value during the trial. Although three cats in Group A and two cats in Group B with serum TT4 concentrations less than 190nmol/l also failed to develop reference range values, it appears that cats with biochemically more severe hyperthyroidism are less likely to benefit from this regimen than those with lower TT4 concentrations.

Some animals in both groups developed variable anorexia or depression. No adverse effects were noted in Group A cats when on the propranolol alone and the number affected was greater, and the degree of the adverse effects were worse, on the higher dosage of potassium iodate and in Group B where the drug was given for the full length of the trial. This strongly suggests that these adverse effects were associated with the potassium iodate and were dose and time related. Concentrated aqueous solutions of potassium iodide may cause salivation, inappetance

or anorexia, proportedly as a result of its unpleasant, brassy taste and administration in gelatin capsules has been recommended to prevent this (Thoday and Mooney, 1992b). Despite this method of administration, variable anorexia or depression still occurred in some cats suggesting that the effect may be systemic rather than local.

Although a number of cats vomited occasionally during treatment, this occurred with almost equal frequency when comparing those on propranolol or potassium iodate alone. Vomiting occurs in approximately 30 per cent of untreated hyperthyroid cats (Thoday and Mooney, 1992b) and may result from a direct action of thyroid hormones on the chemoreceptor trigger zone (Rosenthal, Jones and Lewis, 1976), gastric stasis (Parkin, Nisbet and Bishop, 1982) or rapid overeating (Peterson, Kintzer, Cavanagh *et al.*, 1983), particularly in multicat households (Bernstein, 1984). It is therefore difficult to attribute the vomiting to the potassium iodate therapy.

The cause of the liver disease in the three affected cats was not determined. As well as the adverse effects described above, administration of iodine to cats has been reported to cause muscle spasms, hypothermia, cardiovascular collapse and death (Wilkinson, 1990). We have been unable to trace reports of any direct hepatotoxic effects of iodine in either cats or humans. The clinical, and particularly the histopathological changes, were very different between the three affected cats also making a direct toxic effect less likely. One possible explanation is that the well-recognised liver abnormalities of hyperthyroid cats (Mooney, Thoday and Doxey, 1992) may have been exacerbated by anorexia resulting from iodine administration, leading to hepatic lipidosis and hepatopathy. Hepatic lipidosis has been reported in a hyperthyroid cat (Center, 1994). Another is the development of thyrotoxic storm as described in humans. This is usually of unknown cause but may be associated with increased iodine intake. Thyrotoxic storm results in systolic hypertension and variable gastrointestinal abnormalities including splenomegaly, hepatomegaly, abnormalities in liver function tests and jaundice, leading to high mortality (Wartofsky, 1996). Survival is improved by the administration of propranolol although some workers have reported that standard doses may not prevent storm (Eriksson, Rubenfeld, Garber, *et al.*, 1977). Thyrotoxic storm has not been reported in cats. To our knowledge, propranolol has not been reported as a cause of liver abnormalities in either humans or cats.

Group A cats showed no significant fall in serum TT4 concentrations whilst on propranolol alone. However, there was a significant fall after potassium iodate was introduced. By contrast, serum TT3 concentrations fell significantly over the first 10 day period of treatment in this group and fell significantly further once potassium iodate therapy was begun. The fall in serum TT3 concentrations after beginning propranolol was presumably due to decreased peripheral

conversion of T4 to T3, as in humans so managed (Cooper, Daniels, Ladenson *et al.*, 1982). Effects of propranolol on the peripheral conversion of T4 to T3 in cats does not appear to have been previously reported in cats. However, from data presented in this thesis in Chapter 3, peripheral deiodination of T4 to T3 occurs at similar rates to that in the rat. The fall in both the TT4 and TT3 concentrations after the addition of potassium iodate therapy is likely to have been due to the Wolff-Chaikoff effect. Four (36 per cent) cats in this group had TT4 concentrations within the reference range at the time of surgery. A further cat developed reference range serum TT4 concentrations on day 15, but showed a secondary rise on day 16. Eighty two per cent of cats in this group had reference range serum TT3 concentrations at the time of surgery compared with only 37.5 per cent prior to drug administration.

The reduction in rT3 that occurred in this group during the study is likely to have been a consequence of the fall in serum TT4 which is converted to rT3 in many tissues, combined with reduced *de novo* synthesis.

Group B cats failed to show a significant reduction in serum TT4 concentrations between days 1 and day 20, although overall, there was a significant difference between the days over the treatment period. This was due to the outlier which became more thyrotoxic during the treatment period, as there was a significant fall in TT4 concentrations during the trial period when this data was excluded. Only one cat had a reference range serum TT4 concentration at the time of surgery. Although there was a significant difference in serum TT3 concentrations in Group B cats over the entire treatment period, there was no significant fall during either the first or the second 10 days of the therapeutic regimen. The median serum TT3 concentration on day 20 was lower than at the beginning of the trial. This suggests that the reduced serum TT3 concentrations could not be further lowered by inhibition of TT4 to TT3 conversion as propranolol therapy alone significantly reduced TT3 concentrations in group A. However, 75 per cent of the cats with elevated serum TT3 concentrations in group B on day 1 of the study had reference range TT3 concentrations at the time of surgery and there was no secondary rise in serum TT3 concentrations in the remainder. Interestingly, serum rT3 concentrations were not significantly reduced at any time in this group which had potassium iodate administered for the whole study period, compared with a significant reduction in the cats in group A which only had the potassium iodate for 10 days. The reasons for this are unknown.

The clinical features of thyrotoxicosis improved in all cats in both groups in this study, even in the sole cat in which serum thyroid hormone concentrations increased markedly during therapy.

Feek, Sawers, Irvine *et al.* (1980) showed that a combination of propranolol and potassium iodide (as a source of stable iodine) induced clinical and biochemical euthyroidism in all and nine of 10, human Graves' disease patients respectively, prior to thyroidectomy. In successfully treated patients, both the serum TT3 and TT4 concentrations fell to within their reference ranges within means of 5 and 8 days respectively. There was also a significant fall before surgery, and a transient rise after surgery, of rT3. A secondary rise or 'escape' of thyroid hormones was seen in only two (20 per cent) of patients. The authors suggested that there may be a previously unrecognised synergism between the drugs and that the combination may be the optimal preoperative preparation for patients with Graves' disease.

Although there was no significant difference between the groups, the reduction in heart rates in the group A and B cats in the first halves of the trial respectively is likely to have resulted from different mechanisms. In group A cats, it is likely to have been the result of both β-adrenoceptor blockade and a reduction in serum TT3 concentrations due to the propranolol. In group B cats, heart rates are likely to have been controlled by the rapid reduction of serum thyroid hormone concentrations caused by iodine released from potassium iodate.

These drug combinations induced reference range TT4 concentrations in only five of the 21 cats (23.8 per cent) in this study (four in group A [36.4 per cent] and one in group B [10 per cent]). However, the fall to reference range TT3 concentrations was more marked (89 compared with 75 per cent of those with initially elevated serum concentrations in groups A and B respectively). In the cats which did not achieve values within the respective reference ranges, TT4 and TT3 concentrations were markedly reduced in all but one cat which became more thyrotoxic when given potassium iodate. It has long been recognised that in some humans, the use of stable iodine may exacerbate thyrotoxicosis, both alone (Thompson, Thompson, Brailey *et al.*, 1930) and, rarely, in combination with propranolol (Unger, Couturier, Durez *et al.*, 1981). Whilst this cat had uneventful surgery and outcome, it would be unwise to proceed with potassium iodate treatment if the signs of thyrotoxicosis were clearly worsening.

Another alternative regimen for the medical management of hyperthyroidism in cats, using calcium ipodate, was recently published (Murray and Peterson, 1997). The results are difficult to compare with the present study but 33 per cent of their cats failed to respond and the serum TT4 concentration remained high in all cats, although the serum TT3 concentration fell into the reference range in 66 per cent of the cases. The lack of a fall in TT4 concentrations in a large number of cats is not surprising as ipodate acts on peripheral deiodination of T4 to T3, and not to

reduce *de novo* synthesis of T4. No adverse effects attributable to ipodate therapy were reported in that study.

As previous studies had been carried out on the two drugs individually in humans, it was proposed that the combination of propranolol and potassium iodide may be synergistic in lowering serum thyroid hormone concentrations of patients with Graves' disease (Feek *et al.*, 1980). The separate effects of the two drugs have not been determined in cats and no such conclusions can be made from this study.

Cats in group A appeared to tolerate this regimen better and more cats in this group had reference range TT4 and TT3 concentrations at the time of surgery. Furthermore, Group A cats had a significant reduction in TT3 concentrations over the treatment period whilst Group B cats did not, suggesting the regimen for Group A may be the preferred treatment for clinical cases which cannot tolerate carbimazole. As three cats developed liver pathology, biochemical monitoring of indices of hepatic damage should be undertaken regularly, particularly during the potassium iodate administration phase of the treatment regimen.

In summary, the use of propranolol and potassium iodate may offer an alternative to presurgical treatment with carbimazole in cats that cannot tolerate this drug. The regimen used in group A (propranolol for all 20 days and potassium iodate for the last 10 days) was both the most effective in lowering serum thyroid hormone concentrations and associated with the least adverse effects. As the maximal reduction in both TT4 and TT3 concentrations occurred in the majority of cats in either group, within the first 3 to 5 days of potassium iodate therapy, further studies to determine whether 5 days of potassium iodate therapy would be adequate for stabilisation prior to thyroidectomy may be useful. Further, reduced doses of potassium iodate may be as effective in reducing TT4 and TT3 concentrations and may also prove to have less deleterious effects on feline patients. As the study performed by Birchard, Peterson and Jacobsen (1984) reporting a 75 per cent mortality in surgical feline patients not pretreated medically is now almost 15 years old, the risk of thyroidectomy may be lower than that which this group reported due to the advent of improved monitoring and modern anaesthetics.

The regimen investigated in the present study is unlikely to be of use in the long term medical control of thyrotoxicosis in cats as it would be expected that cats would eventually 'escape' from the effects of potassium iodate as has been described in humans.

9.00 CONCLUDING REMARKS

Hyperthyroidism caused by toxic nodular goitre, is the commonest endocrine disease of the domestic cat. Despite this, little is known about feline thyroid physiology, pathophysiology, nor the pathogenesis of the disease.

This thesis has examined several aspects regarding the pathogenesis and management of hyperthyroidism and thyroid hormone deiodination in the domestic cat. The main objectives of this thesis were to examine a number of possibilities regarding the pathogenesis of feline hyperthyroidism. These included determining the possible existence and significance of thyroid growth stimulating immunoglobulins and TSHR mutations in the pathogenesis of feline nodular goitre. Selenoenzyme expression in the feline thyrocyte and selenium status in cats was also studied to determine their possible role in the pathogenesis of this important endocrine disease. In addition to this, thyroid hormone deiodination in the domestic cat was investigated since this reaction is catalysed by selenoenzymes. Finally, a new method for pre-surgical stabilisation was examined as a possible alternative treatment in cats which cannot tolerate carbimazole.

The work in this thesis has allowed the following conclusions to be reached.

1. While feline liver and kidney express IDI at similar concentrations, and the feline enzyme is able to metabolise T4 at a similar rate to that of rats, feline IDI is unusual in that it has little ability to metabolise reverse triiodothyronine which is the preferred substrate for this enzyme in rats and humans. Additionally, unlike all other carnivores and omnivores studied thus far, cats were not found to express thyroidal IDI. The sequence of the feline enzyme would be of particular interest.

2. Brown's group (1992) reported the existence of thyroid growth stimulating immunoglobulins in cats with thyrotoxicosis. We have been unable to confirm her findings in the most appropriate cell model (feline thyrocytes). Using feline thyrocytes, Chinese hamster ovary cells expressing human TSHR (JPO9) and the rat thyrocyte cell line FRTL-5, no populations of immunoglobulins were detectable in the sera of hyperthyroid cats which stimulated cAMP production, displaced TSH binding from its receptor or induced growth when compared to euthyroid cats. Similar results were found using unpurified sera. These results indicate that no humoral factor is present in the sera of thyrotoxic cats that could be responsible for the disease.

3. As many selenoenzymes are involved in regulating thyroid hormone production (GPX), metabolism (ID) and cell growth (TR), we investigated the selenoenzyme profile of both normal and adenomatous feline thyrocytes and the selenium status in cats from four different regions. TR was found to be overexpressed in a number of adenomatous thyrocyte preparations. This may reflect the increased growth characteristics of these cells, or, in addition, the enzyme may help to protect the adenomatous thyrocyte from oxidative damage by H_2O_2 during thyroid hormone production. Alternatively, it may be that increased TR expression may in turn promote the disease. Simultaneous experiments characterising selenoenzyme and growth characteristics may help to determine the significance of enhanced TR expression.

4. The plasma selenium status of cats from areas with high (Edinburgh, U.K. and Sydney, Eastern Australia) and low (Greve, Denmark and Perth, Western Australia) incidences, respectively, of hyperthyroidism were not significantly different from each other. Cats, however, have plasma selenium concentrations and red blood cell glutathione peroxidase activities which are approximately 10 times that of selenium replete rats and humans. High selenium status of cats may increase their risk of thyrotoxicosis but other factors may not allow the expression of this disease in certain areas (e.g. Perth and Greve). It would, therefore, be interesting to examine other known risk factors of feline hyperthyroidism (indoor lifestyle, eating canned food and the use of flea-sprays, herbicides and pesticides) and to determine longevity of cats in these four regions. The possible link between iodine and selenium intake in domestic cats may also provide further evidence for dietary triggers of this disease.

5. Our work could find no evidence of activating mutations in the TSH receptor of affected cats. In 11 hyperthyroid cats, the DNA for the TSHR region between codons 480 and 640 (the most common site for somatic mutations in human toxic nodular goitre) were not found to contain any such mutations.

6. The mainstay of presurgical treatment for hyperthyroidism is the use of carbimazole, a drug which is poorly tolerated by approximately 8 per cent of affected cats. A combination of propranolol and potassium iodate was found to normalise heart rates, serum T3, and to a lesser extent T4 concentrations in a significant number of cats and may be used as an alternative presurgical treatment in those cats which cannot tolerate carbimazole.

In conclusion, this thesis has provided evidence that:

1. Growth stimulating immunoglobulins do not appear to be an important part of the pathogenesis of feline hyperthyroidism.
2. Unlike human toxic nodular goitre, somatic TSHR mutations appear to be an uncommon cause of feline hyperthyroidism.
3. Feline thyroid hormone metabolism is different to that in other species. Cats appear to lack the ability to metabolise rT3 by deiodination and (unlike other carnivores/omnivores investigated) lack expression of thyroidal IDI. However, the majority of plasma T3 is likely to be generated by the peripheral deiodination of T4 as in other species.
4. The expression of selenoenzymes in some adenomas appears to be different to that of normal feline thyrocytes and may be an important part of the growth and functional characteristics of thyroid adenomas.
5. Cats appear to have extremely high blood selenium status in many areas of the world. The implications for feline health may be dramatic and require further investigation.
6. The use of beta-blocking drugs such as propranolol with the combination of stable iodine may provide an important pre-surgical alternative to carbimazole.

Further studies that would be of interest would be to clone feline IDI to determine the important sequence differences between cats and other species and to examine peripheral deiodination of rT3 in this species in more detail. Whilst this thesis was in preparation, a collaborative study between our group and a group in France was carried out to investigate the peripheral metabolism of ^{14}C-T4 *in vivo* in cats (compared to rabbits) to confirm our *in vitro* data that shows feline IDI can deiodinate T4. It would be of interest to examine G-protein expression and investigate the possibility for G-protein mutations which may be responsible for this disease and possibly extend the cloning of the feline TSHR to determine if somatic mutations in other parts of the receptor may be responsible for feline thyrotoxicosis. Multicentre epidemiological studies from areas with varying incidences of thyrotoxicosis would be of interest to examine the possible risk factors already determined to see if there is a difference between these areas. The iodine status of cats from around the world may also be of help in determining dietary risk factors.

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11.00 PUBLICATIONS CURRENTLY ARISING FROM THIS THESIS

PUBLISHED CONFERENCE PROCEEDINGS

Foster, D.J., Thoday, K.L. & Beckett, G.J. 1998. Thyroid hormone deiodination in the thyroid and liver of the domestic cat. *Journal of Endocrinology, Abstract supplement, British Endocrine Societies 17th Joint Meeting* **156**: P301.

PEER-REVIEWED PUBLICATIONS

Foster, D.J. & Thoday, K.L. The use of propranolol and potassium iodate in the pre-surgical management of hyperthyroid cats. *Journal of Small Animal Practice* , accepted for publication.

Pearce, H.S., **Foster, D.J.,** Imrie, H., Myerscough, N., Beckett, G.J., Thoday, K.L. & Kendall-Taylor, P. 1997. Mutational analysis of the thyrotropin receptor gene in sporadic and familial feline thyrotoxicosis. *Thyroid* **7**: 923 - 927.

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P301 THYROID HORMONE DEIODINATION IN THE THYROID AND LIVER OF THE DOMESTIC CAT.

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The iodothyronine deiodinases are selenoenzymes that play an important role in the peripheral conversion of thyroxine (T4) to triiodothyronine (T3), reverse triiodothyronine (rT3) and diiodothyronines. In most species around 80% of circulating T3 originates from the 5' mono-deiodination of T4 in the liver and kidney by type I iodothyronine deiodinase (IDI). In many omnivorous species, such as rodents and humans, thyroidal IDI may also provide an important source of circulating T3 since the liver and thyroid of these species express high activities of IDI. Little or no thyroidal IDI is expressed by herbivores (e.g. deer, llama, goat, sheep, cattle) but high expression of IDI is found in the liver of these species. Type II iodothyronine deiodinase (IDII) also carries out 5' monodeiodination of T4 to produce T3 but this enzyme usually contributes little to circulating T3. IDII is not expressed by liver but is found mainly in brown adipose tissue and brain.

Hyperthyroidism is a common disease in the cat but little is known about the process of thyroid hormone deiodination in this species. We have assessed thyroid hormone deiodination in the liver and thyroid of the cat using ^{125}I-rT3 as a substrate. We found we were unable to detect any deiodinase activity in the thyroid of two euthyroid cats and three thyrotoxic cats. The deiodinase activity in liver homogenates prepared from three cats was only approximately 10% of that found in rat liver. In cat liver, deiodinase activity could not inhibited the addition of aurothioglucose (1μM - 10mM) or propylthiouracil (1μM - 10 mM), suggesting this enzyme was not IDI but rather IDII. These results suggest that in the cat, hepatic and thyroidal deiodination plays only a minor role in generating the circulating pool of T3. In the cat circulating T3 may be derived from predominantly *de novo* thyroidal synthesis or by deiodination in other tissues.

THE USE OF PROPRANOLOL AND POTASSIUM IODATE IN THE PRE-SURGICAL MANAGEMENT OF HYPERTHYROID CATS

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ABSTRACT

A prospective study was carried out using a combination of propranolol and potassium iodate to assess whether there were beneficial effects in preparing hyperthyroid cats for surgical thyroidectomy. Group A (n = 11) received propranolol from days 1 to 10 followed by propranolol and potassium iodate from days 11 to 20 whereas group B (n = 10) received the reverse regimen. Blood samples were taken daily for subsequent determination of serum total L-thyroxine (TT4), L-triiodothyronine (TT3) and reverse T3 (rT3) concentrations. The signs of hyperthyroidism improved in all cats over the treatment period. At surgery, in group A, 36 per cent of the cats had reference range serum TT4 concentrations while 89 per cent with initially elevated TT3 concentrations had reference range concentrations. In group B, 10 per cent of the cats had a reference range TT4 concentration while 75 per cent with initially elevated TT3 concentrations had reference range concentrations. The drug regimen used in group A was better tolerated and more effective and offers an alternative prior to thyroidectomy in cats that cannot tolerate carbimazole.

INTRODUCTION

The induction of euthyroidism by prior short-term medical therapy dramatically reduces peri-surgical mortality in hyperthyroid cats (Birchard and others, 1984). The most commonly used drug to treat cats prior to surgical thyroidectomy in the UK is the thioglyoxaline carbimazole. However, adverse effects (vomiting, anorexia and depression) requiring withdrawal of the drug, occur in approximately 8 per cent of treated cats (Mooney and others, 1992) and these cats usually go to surgery still thyrotoxic.

It has previously been shown in humans with hyperthyroidism caused by Graves' disease, that a combination of propranolol and potassium iodide (as a source of stable iodine) induced euthyroidism before surgical thyroidectomy (Feek and others, 1980). A pharmaceutical preparation of potassium iodide is no longer available in the UK and potassium iodate is now being used in its place.

The aim of this prospective study was to determine whether a combination of propranolol and potassium iodate might be an effective method of preparing cats for surgical thyroidectomy.

MATERIALS AND METHODS

Clinical Material

The clinical material comprised a series of 23 hyperthyroid cats presented to the Small Animal Clinic of The University of Edinburgh's Royal (Dick) School of Veterinary Clinical Studies. A tentative diagnosis of hyperthyroidism was made on the basis of compatible historical and physical features and confirmed by the presence of elevated serum total L-thyroxine (TT4) concentrations in all cases.

Before admission to the study, blood was collected by jugular venepuncture for haematological examinations and determination of a 13 parameter biochemical profile. Urine was collected by cystocentesis. Animals with identified concurrent non-thyroidal diseases were excluded from the study. After the informed consent of their owners, animals were were assigned alternately to one of two groups (A and B) and hospitalised for the duration of the study.

Study Protocols

Group A cases received propranolol (Inderal, Zeneca) alone at 2.5mg/cat every eight hours from days 1 to 10 inclusive. The heart rate was determined each morning. If an animal's heart rate exceeded 200 beats/minute on day 4, the propranolol dosage was increased to 5mg/cat every eight hours and if it still exceeded this rate on day 7, to 7.5 mg/cat every eight hours for the remainder of the 10 days. Subsequently, the same dose of propranolol and, initially, 42.5mg potassium iodate (equivalent to 25mg free iodine, Potassium Iodate tablets 85mg, Cambridge Self-Care Diagnostics) were administered every eight hours from days 11 to 20 until the time of surgery. The potassium iodate tablets were placed in gelatin capsules. Surgery was carried out on day 21. Potassium iodate was stopped immediately before thyroidectomy while

propranolol was continued post-operatively for a further three days.

Group B cases were treated in the same way with the exception that they received potassium iodate alone from days 1 to 10 inclusive followed by potassium iodate and propranolol in combination from days 11 to 20 inclusive, with propranolol alone being continued post-operatively for a further three days.

Potassium iodate at the above dose appeared to cause an unacceptable degree of adverse effects (intermitent anorexia and vomiting, and mild depression) in some cats. Consequently, the dosage was reduced to 21.25mg (equivalent to 12.5mg free iodine) and no further adverse effects were observed.

Cats were bled daily from day 1 to day 20 inclusive by jugular venepuncture. Two ml whole blood were collected in a plain tube. Serum was harvested and stored at -40°C for subsequent assay of TT4, total L-triiodothyronine (TT3) and 3,3',5' triiodothyronine (reverse triiodothyronine, rT3), which was carried out in respective batch modes. Complete blood counts were carried out at five day intervals on each cat to ensure that repeated blood sampling did not result in values outside the respective reference ranges. Serum potassium concentrations were determined in three cats which became anorexic on days 15 (one in group A and one in group B, on 42.5 and 21.25mg potassium iodate respectively) and on day 17 in one cat in group A on 42.5mg potassium iodate.

Laboratory Studies

Complete blood counts, serum biochemical tests and urine analyses were carried out by standard techniques.

Serum TT4 concentrations were determined using a double antibody radioimmunoassay (RIA) technique (DPC TT4). Serum TT3 concentrations were determined using a double antibody RIA technique (Amerlex MT3). Serum rT3 concentrations were determined using a polyethylene glycol separation radioimmunoassay technique (Biostat Diagnostics).

The serum TT4 and TT3 assays were specifically validated for use with cat serum. The accuracy of the assays was assessed by determining the recovery of known amounts of T4 or T3 which had been added to the diluent used in the standard production. For TT4 and TT3, the mean recovery of low and high concentrations in six experiments per analyte was 104.9 per cent and 97.9 per cent respectively. The specificity of the assays was demonstrated by dilutional parallelism. The precision of the assays and an acceptable degree of drift were assessed by the inclusion of low, medium and high pools in each assay. The mean within-assay coefficients of variation for TT4 and TT3 were 4 per cent and 8 per cent respectively and the mean between-assay coefficients of variation for TT4 and TT3 were 6.6 per cent and 6.0 per cent respectively. The working ranges of the assays were 4.0 to 300.0 nmol/l for TT4 (reference range 19.0 to 65.0 nmol/l) and 0.20 to 12.00 for TT3 (reference range 0.90 to 3.10 nmol/l).

The RIA for rT3 was not specifically optimised and validated for feline serum but each cat acted as its own control during the study.

Statistical Analyses

The Friedman test was used to examine overall differences in thyroid hormone concentrations between days 1 to 20 within the treatment groups. The Wilcoxon Signed Rank test was used to test for differences in heart rates and thyroid hormone concentrations between days 1, 11 and 20 within groups. The Mann-Whitney U test was used to examine differences in heart rates and thyroid hormone concentrations between days 1, 11 and 20 between groups. With all tests, a p value of < 0.05 was considered significant.

RESULTS

Twenty-three cats were admitted to the trial. One cat had to be withdrawn from group A on day 8 due to the development of congestive cardiac failure and was successfully treated with carbimazole followed by surgical thyroidectomy. One cat in Group A died suddenly on day 8 of treatment due to an hepatic infarct.

Twenty-one cats completed the trial with 11 in Group A and 10 in Group B (Table 1), although one cat in each group died on induction of anaesthesia. There were 16 Domestic Shorthaired and **five** Domestic Longhaired animals with 15 being ovariohysterectomised females and six castrated males (three castrated males being present in each of the groups). The median age was 13 years (range 7 to 16 years). There was no significant difference in the age of the cats between groups A (median 13 years; range 7 to 16 years) and B (median 12.5 years, range 9 to 15 years) ($p = 0.8603$).

Group A Cats

In this group, four cats were treated with 42.5mg three times daily and seven were treated with 21.25mg of potassium iodate three

times daily. The only cat in the study which required 7.5mg propranolol three times daily was in this group.

Clinical Response

The drugs were well-tolerated by most cats. No adverse effects were noted when the cats were taking the propranolol alone. However, intermittent anorexia and mild depression were seen in six of the 11 cats in this group once potassium iodate was introduced. Of the six cats that developed inappetance, three were on 42.5 mg three times daily and three were on 21.25 mg of potassium iodate three times daily. Six cats vomited at some point during the trial, two of which which had vomiting as part of their previous history with four cats vomiting whilst on propranolol alone.

Table 2 shows the data for the heart rates of the 11 cats in group A.

Heart rates were well-controlled by the propranolol alone and fell significantly from days 1 to 11 ($p = 0.0040$), over the entire treatment period (days 1 to 20) ($p = 0.0040$) but not from days 11 to 20 ($p = 0.2490$).

Thyroid Hormone Concentrations

Fig. 1 shows the serum TT4 concentrations of the cats in group A over the study period and the data are summarised in Table 3.

Of the 11 cats in this group, all had elevated TT4 concentrations on day 1 of the study. Of these, four (36 per cent) had normal TT4 concentrations on day 20 of the study. The serum TT4 concentrations on day 20 were significantly lower than those on day 1 ($p = 0.0370$), and there was a significant difference between the days over the treatment period ($p = 0.0000$). There was no significant difference in TT4 concentrations between the first and last days of propranolol treatment alone (day 1 compared to day 11) ($p = 0.3070$). but the TT4 concentrations fell significantly between the first and last days of combined propranolol and potassium iodate treatment (day 11 compared to day 20) ($p = 0.0370$).

Fig. 2 shows the serum TT3 concentrations of the cats in group A over the study period and the data are summarised in Table 3.

Of the 11 cats, nine (82 per cent) had elevated total T3 concentrations at the time of diagnosis and eight of these (89 per cent) had reference range TT3 concentrations at the time of surgery. The serum TT3 concentrations on day 20 were significantly lower than those on day 1 ($p = 0.0040$), and there was a significant difference between the days over the treatment period ($p = 0.000$). There was a significant fall in the serum TT3 concentrations between day 1 and day 11 ($p = 0.0370$) and between days 11 and 20 ($p = 0.0040$).

The data for serum rT3 concentrations of the 11 cats in group A are summarised in Table 3.

The serum rT3 concentrations on day 20 were significantly lower than on day 1 ($p = 0.0040$). The rT3 concentrations did not change significantly from days 1 to 11 ($p = 0.1850$), but fell significantly from days 11 to 20 ($p = 0.0090$).

Group B Cats

In this group, 4 cats were treated with 42.5 mg three times daily and 6 were treated with 21.25 mg of potassium iodate three times daily.

Clinical Response

This regimen seemed less well-tolerated by the cats generally. Anorexia and depression were noted in four cats (three on 42.5 mg potassium iodate three times daily and one on 21.25 mg potassium iodate three times daily). Jaundice developed in two cats being given 42.5 mg potassium iodate towards the end of the treatment period (days 16 and 18 respectively). Both completed the study. One underwent surgical thyroidectomy and liver biopsy via exploratory laparotomy. Histopathological examination revealed hepatic lipidosis. The cat survived for 7 days but subsequently died due to complications of its liver disease. A further cat in this group also died on induction of anaesthesia. Post-mortem examination revealed no abnormalities in this cat other than bilateral thyroid adenomatous hyperplasia. Eight out of 10 cats vomited during the trial, five of which were presented for vomiting as part of their previous history.

The data for heart rates of the 10 cats in group B are summarised in Table 2.

Heart rates were well controlled during the treatment period and fell significantly from days 1 to 20 ($p = 0.0060$), from days 1 to 11 ($p = 0.0060$) and from days 11 to 20 ($p = 0.0210$).

Thyroid Hormone Concentrations

Fig. 3 shows the serum TT4 concentrations of the 10 cats in group B over the study period and the data are summarised in Table 3.

Of the 10 cats in this group, all had elevated serum TT4 concentrations on day 1 of the study. Of these, one (10 per cent) had a normal serum TT4 concentration on day 20 of the study, although a further three cats were transiently euthyroid (during days 4 to 15 inclusive [two cats] and days 16 and 17 [one]).

The serum TT4 concentrations on day 20 were not significantly lower than those on day 1 ($p = 0.2340$), although there was a significant difference between the days over the treatment period ($p = 0.002$). TT4 concentrations fell significantly between the first and last days of potassium iodate treatment alone (day 1 compared to day 11) ($p = 0.041$), but not between the first and last days of combined propranolol and potassium iodate treatment (day 11 compared to day 20) ($p = 0.2800$).

Fig. 4 shows the serum TT3 concentrations of the cats in group B over the study period and the data are summarised in Table 3.

Of the 10 cats in group B, four (forty per cent) had elevated TT3 concentrations on day 1 of the study. Of these four cats,. three (75 per cent) had normal T3 concentrations at the time of surgery. The serum TT3 concentrations on day 20 were not significantly lower than those on day 1 ($p = 0.1240$), although there was a significant difference between days over the treatment period ($p = 0.002$). There was no significant fall in TT3 concentrations from days 1 to 11 ($p = 0.1390$) or from days 11 to 20 ($p = 0.7220$).

The data for serum rT3 concentrations of cats in group B are summarised in Table 3.

The serum rT3 concentrations were not significantly different between days 1 and 20 ($p = 0.6360$), between days 1 and 11 ($p = 0.4760$) or 11 and 20 ($p = 0.2860$).

Complete Blood Counts

No significant abnormalities attributable to repeated blood sampling were seen in any parameter in any cat in the study.

Serum Potassium Concentrations

The three anorexic cats which had serum potassium concentrations determined all showed values within the reference range (4.00 - 5.00 nmol/l).

Comparison Of Group A And Group B Cats

Heart Rates

The data comparing the heart rates of the cats in Groups A and B are summarised in Table 3.

There was no significant difference in heart rates between group A and B cats at days 1, 11 or 20 ($p = 0.9439$, 0.1809 and 0.3981 respectively).

Thyroid Hormone Concentrations

The data comparing the serum TT4, TT3 and rT3 concentrations of the cats in Groups A and B are summarised in Table 3.

For serum TT4 concentrations, there was no significant difference between group A and B cats at days 1, 11 or 20 ($p = 0.3418$, 0.6472 and 0.3069 respectively).

For serum TT3 concentrations, there was no significant differences between group A and B cats at days 1, 11 or 20 ($p = 0.5035$, 0.1300 and 0.3823 respectively).

For serum rT3 concentrations, there was no significant difference between group A and B cats at days 1, 11 or 20 ($p = 1.000$, 0.3847, and 0.4033 respectively).

Liver Pathology

Histological examination of the liver was carried out on three cats, identified by the numbers in Table 1. All were taking 42.5mg of potassium iodate three times daily. Cat 3 was in Group A and was the sole cat taking 7.5 mg propranolol three times daily from day 7 of the study. The cat died on anaesthetic induction. Liver pathology showed hepatocellular necrosis, with minimal periportal lymphocyte and neutrophil infiltration, indicative of mild cholangitis. The liver changes were not considered severe enough to have contributed to the cat's death.

Two (cats 14 and 15) were in Group B and were taking 5mg propranolol three times daily when they developed jaundice. Cat 14 was improving at the end of the trial when it had an ultrasound-guided **needle** liver biospy. Liver histology from this cat showed mild fatty degeneration together with acute cholangitis. Cat 15 had a liver biopsy at the time of thyroidectomy. The biopsy results showed focal fatty nodules and areas of fatty degeneration of cytoplasm indicative of hepatic lipidosis. However, at post-mortem examination, histological examination showed more chronic degenerative changes without significant associated fatty abnormalities.

DISCUSSION

Feline hyperthyroidism (thyrotoxicosis) is the most commonly diagnosed endocrine disease of cats (Thoday and Mooney, 1992). Despite a number of treatment options, the most reliable in the medium to long-term for the practitioner is surgical thyroidectomy. As hyperthyroid cats are poor anaesthetic risks (Peterson and others, 1983) it is recommended that euthyroidism is induced prior to surgery (Birchard and others, 1984). The drugs of

choice are the thioglyoxalines carbimazole (NeoMercazole, Roche) available in Europe and methimazole (Tapazole, Eli Lilly) available in the U.S.A.

Mild clinical adverse reactions (anorexia, vomiting and lethargy) requiring drug withdrawal occur in approximately 8 per cent and 15 per cent of cats treated carbimazole and methimazole respectively (Peterson and others, 1988; Mooney and others, 1992). Serious adverse effects, such as thrombocytopenia and agranulocytosis, have been reported in up to 3 per cent of cats treated with methimazole (Peterson and others, 1988) but not in cats treated with carbimazole.

Other drugs that have been used to treat feline hyperthyroidism include stable iodine (^{127}I) and the beta-adrenoceptor blocking agents.

Stable iodine was the earliest agent used effectively to treat human thyrotoxicosis. Large doses dramatically decrease thyroid hormone synthesis (the Wolff-Chaikoff effect) by reducing peroxidase-catalysed organification of iodide (Wolff and Chaikoff, 1948). Thyroid hormone release is also decreased as a result of inhibition of thyroglobulin endocytosis. Unfortunately, the failure of thyroid function of most human patients to normalise (Emerson and others, 1975) and rapid escape from its inhibitory control (Cooper, 1991) mean that iodine cannot be used as sole therapy for long term management of thyrotoxicosis.

It is currently believed that an excess of thyroid hormones causes an increase in the number of beta-adrenoceptors or an amplification of the adrenergic signal at the cell membrane level (Trepanier, 1990) and that many of the signs of hyperthyroidism are due to subsequently enhanced catecholamine activity with increased sympathetic drive. Thus, the beta-adrenoceptor blocking drugs very effectively ameliorate many of these signs and some studies (Feely and Peden, 1984; Lennquist and others, 1985; Alderberth and others, 1987) have reported them to be as effective as the thioureylenes in the pre-surgical management of human patients awaiting thyroidectomy. However, the negative nitrogen balance, increased cardiac output and elevated rate of oxygen consumption of thyrotoxicosis are seldom normalised (O'Malley and others, 1982) and patients remain hyperthyroid.

Propranolol, the beta-adrenoceptor blocking drug most frequently used in hyperthyroid cats, has been employed as the sole agent for presurgical stabilisation (Carlson, 1986). It is a non-selective $beta_1$- and $beta_2$-adrenoceptor blocking agent with no direct effects on the thyroid gland. However, like some other beta-adrenoreceptor blockers, propranolol decreases peripheral monodeiodination of T4 to T3 (Feely and Peden, 1984). However, serum TT3 concentrations fall by a maximum of 30 per cent, serum TT4 concentrations are usually unaffected and euthyroidism is not restored (Lotti and others, 1977; Wiersinga and Touber, 1977).

It is clear, therefore, that an alternative method of preparation for surgery is required for a relatively small percentage, but, because of the high incidence of the disease, a very significant number, of hyperthyroid cats.

Feek and others (1980) showed that a combination of propranolol and potassium iodide (as a source of stable iodine) induced clinical and biochemical euthyroidism in all and nine of 10, human Grave's patients respectively prior to thyroidectomy. In successfully treated patients, both the serum TT3 and TT4 concentrations fell to their reference ranges within means of 5 and 8 days respectively. There was also a significant fall before surgery, and a transient rise after surgery, of rT3. A secondary rise or 'escape' of thyroid hormones was seen in only two (20 per cent) of patients. The authors suggested that there may be a previously unrecognised synergism between the drugs and that the combination may be the optimal preoperative preparation for patients with Grave's disease.

Pharmaceutical preparations of both potassium iodide and iodine are currently unavailable in the UK. Potassium iodate is now used as the source of stable iodine in human medicine.

A condition comparable to Grave's disease has not been recorded in cats. The causal pathological change is commonly termed thyroid adenoma (adenomatous hyperplasia), which closely resembles that seen in human toxic nodular goitre. Apart from a pilot study by one of us (Thoday, 1986), to our knowledge there are no previous reports of the use of propranolol and stable iodine to reduce plasma thyroid hormone concentrations prior to surgery in feline toxic nodular goitre.

In the human Grave's disease study, patients were treated with 60mg potassium iodide (yielding 33mg free iodine) three times daily. A proportionately higher dose of free iodine (25mg three times daily) was initially administered to the cats in this study to ensure that any potential effect would definitely be induced. Although this dose was subsequently reduced to 12.5mg three times daily, this is unlikely to have had any effect on the outcome of the study as 3.3mg free iodine three times daily has previously been shown to have significant inhibitory effects on thyroid hormone production in cats (Thoday, 1986). The dose of

propranolol used (2.5 to 7.5 mg per cat three times daily) has been shown to be effective in blocking the increased sympathetic effects of hyperthyroidism in most affected cats. Efficacy was determined by reduction of heart rate to below 200 beats per minute. This has been reported to be an appropriate clinical method of determining adequate ß-adrenergic blockade in cats (Jacobs and others, 1997).

In this study two groups were chosen to examine initially the independent action of propranolol and potassium iodate and then to investigate their combined effect. As thyroid hormone concentrations may fluctuate markedly in hyperthyroid cats not receiving any form of therapy (Peterson and others, 1987) and, in some cats, TT3 concentrations may fall rapidly when treated with potassium iodate and propranolol (Thoday, 1986) blood sampling was carried out daily. No adverse effects on haematological parameters from this protocol were noted.

The cases included in the trial comprised a combination of Domestic Short and Longhaired cats, with a median age in the two groups of 12.5 and 13 years. This part of the signalment is highly comparable to previously reported series of hyperthyroid cats (Peterson and others, 1983; Thoday and Mooney, 1992). There were 2.5 times the number of ovariectomised females than castrated males. However, this is likely to have arisen by chance as there is no reported sex predisposition for feline hyperthyroidism, either in our clinic (Thoday and Mooney, 1992) or elsewhere (Hoenig and others, 1982; Peterson and others, 1983). As the study was designed to evaluate the effect of the drugs on serum thyroid hormone concentrations and as concurrent diseases may affect these, animals with any identified non-thyroidal diseases were excluded from the study.

At the beginning of the trial, all cats had elevated TT4 concentrations, while a number in both groups had reference range TT3 concentrations. The latter has been well recorded in hyperthyroid cats and may result from within or between day variations in mildly affected animals or the effects of non-thyroidal illness (Peterson and others, 1987; Peterson & Gamble, 1988). As cats with non-thyroidal illness were, as far as could be determined, excluded from the study, the reference range TT3 values in this study probably resulted from intra or inter-daily variations which is the far commoner cause in feline thyrotoxicosis (Thoday and Mooney, 1992). No cat that had a TT4 concentration at diagnosis of more than 190nmol/l developed a reference range value during the trial. Although three cats in Group A and two cats in Group B with serum TT4 concentrations less than 190nmol/l also failed to develop reference range values, it appears that cats with biochemically more severe hyperthyroidism are less likely to benefit from this regimen than those with lower TT4 concentrations.

Some animals in both groups developed variable anorexia or depression. No adverse effects were noted in Group A cats when on the propranolol alone and the number affected was greater and the degree of the adverse effects were worse on the higher dosage of potassium iodate and in Group B where the drug was given for the full length of the trial. This strongly suggests that these adverse effects were associated with the potassium iodate and were dose and time related. Concentrated aqueous solutions of potassium iodide may cause salivation, inappetance or anorexia, proportedly as a result of its unpleasant, brassy taste and administration in gelatin capsules has been recommended to prevent this (Thoday and Mooney, 1992). Despite this method of administration, variable anorexia or depression still occurred in some cats suggesting that the effect may be systemic rather than local.

Although a number of cats vomited occassionally during treatment, this occurred with almost equal frequency when comparing those on propranolol or potassium iodate alone. Vomiting occurs in approximately 30 per cent of untreated hyperthyroid cats (Thoday and Mooney, 1992) and may result from a direct action of thyroid hormones on the chemoreceptor trigger zone (Rosenthal and others, 1976), gastric stasis (Parkin and others, 1982) or rapid overeating (Bernstein, 1984). It is therefore difficult to attribute the vomiting to the potassium iodate therapy.

The cause of the liver disease in the three affected cats was not determined. As well as the adverse effects described above, administration of iodine to cats has been reported to cause muscle spasms, hypothermia, cardiovascular collapse and death (Wilkinson, 1990). We have been unable to trace reports of any direct hepatotoxic effects in either cats or humans. The clinical, and particularly the histopathological changes were very different between the three affected cats also making a direct toxic effect less likely. One possible explanation is that the well-recognised liver abnormalities of hyperthyroid cats (Mooney and others, 1992) may have been exacerbated by anorexia resulting from iodine administration, leading to hepatic lipidosis and hepatopathy. Hepatic lipidosis has been reported in a hyperthyroid cat (Center, 1994). Another is the development of thyrotoxic storm

as described in humans. This is usually of unknown cause but may be associated with increased iodine intake. Thyrotoxic storm results in systolic hypertension and variable gastrointestinal abnormalities including splenomegaly, hepatomegaly, abnormalities in liver function tests and jaundice, leading to high mortality (Wartofsky, 1996). Survival is improved by the administration of propranolol although some workers have reported that standard doses may not prevent storm (Eriksson and others, 1977). Thyrotoxic storm has not been reported in cats. To the authors' knowledge, propranolol has not been reported as causing liver abnormalities in either humans or cats.

Group A cats showed no significant fall in TT4 concentrations whilst on propranolol alone. However, there was a significant fall after potassium iodate was introduced. By contrast, TT3 concentrations fell significantly over the first 10 day period of treatment in this group and fell significantly further once potassium iodate therapy was begun. The fall in TT3 concentrations after beginning propranolol was presumably due to decreased peripheral conversion of T4 to T3, as in humans so managed (Cooper and others, 1982) whilst the fall in both the TT4 and TT3 concentrations after the addition of potassium iodate therapy is likely to have been due to the Wolff-Chaikoff effect. Four (36 per cent) cats in this group had TT4 concentrations within the reference range at the time of surgery. A further cat developed reference range serum TT4 concentrations on day 15, but showed a secondary rise on day 16. Eighty two per cent of cats in this group had reference range TT3 concentrations at the time of surgery compared with only 37.5 per cent prior to drug administration.

Reverse T3, a metabolically inactive product, results from deiodination of T4 in its inner ring. In humans, rT3 is almost entirely derived from extrathyroidal deiodination of T4 (Chopra, 1976), the remainder being synthesised *de novo* in the thyroid gland. The situation in cats is unknown. The reduction in rT3 that occurred in this group during the study is likely to have been a consequence of the fall in serum TT4 concentrations.

Group B cats failed to show a significant reduction in serum TT4 concentrations between days 1 and day 20, although there was a significant difference between the days over the treatment period. This was probably due to the rapid fall in TT4 concentrations at the beginning of potassium iodate therapy with no further significant fall following this. Only one cat had a reference range serum TT4 concentration at the time of surgery. Although there was a significant difference in TT3 concentrations in Group B cats over the entire treatment period, there was no significant fall during the first 10 days of potassium iodate therapy or combination therapy. This suggests that the reduced serum TT3 concentrations could not be further lowered by inhibition of TT4 to TT3 conversion as propranolol therapy alone significantly reduced TT3 concentrations in group A. However, 75 per cent of the cats with elevated serum TT3 concentrations in group B on day 1 of the study had reference range TT3 concentrations at the time of surgery and there was no secondary rise in serum TT3 concentrations in the remainder. Interestingly, serum rT3 concentrations were not significantly reduced at any time in this group which had potassium iodate administered for the whole study period, compared with a signigicant reduction in the cats in group A which only had the potassium iodate for 10 days. The reasons for this are unknown.

The clinical features of thyrotoxicosis improved in all cats in both groups in this study, even in the cat in which serum thyroid hormone concentrations increased during therapy.

Although there was no significant difference between the groups, the reduction in heart rates in the group A and B cats in the first halves of the trial respectively will have resulted from different mechanisms. In group A cats, it would have been the result of both β-adrenoceptor blockade and a reduction in serum TT3 concentrations due to the propranolol. In group B cats, heart rates would have been controlled by the rapid reduction of serum thyroid hormone concentrations caused by iodine released from potassium iodate.

These drug combinations induced reference range TT4 concentrations in only five of the 21 cats (23.8 per cent) in this study (four in group A [36.4 per cent] and one in group B [10 per cent]. However, the reduction to reference range TT3 concentrations was far higher (89 and 75 per cent of those with initially elevated serum concentrations in groups A and B respectively). In the cats which did not achieve the respective reference ranges, TT4 and TT3 concentrations were markedly reduced in all but one which became more thyrotoxic when given potassium iodate. It has long been recognised that in some humans, the use of iodides exacerbates thyrotoxicosis, both alone (Thompson and others, 1930) and rarely in combination with propranolol (Unger and others, 1981). Whilst this cat had uneventful surgery and outcome it would be unwise to proceed with potassium iodate treatment if the signs of thyrotoxicosis were clearly worsening.

Another alternative regimen for the medical management of hyperthyroidism in cats, using

calcium ipodate, was recently published (Murray and Peterson, 1997). The results are difficult to compare with the present report but 33 per cent of their cats failed to respond and the serum TT4 concentration remained high in all cats, although the serum TT3 concentration fell into the reference range in 66 per cent of the cases. From these parameters, as a group, the animals in the present study responded more satisfactorily. However, no adverse effects attributable to ipodate therapy were reported in that study.

As previous studies had been carried out on the two drugs individually in humans, it was proposed that the combination of propranolol and potassium iodide may be synergistic in lowering serum thyroid hormone concentrations of patients with Grave's disease (Feek and others, 1980). The separate effects of the two drugs have not been determined in cats and no such conclusions can be made from this study.

Cats in group A appeared to tolerate this regimen better and more cats in this group had reference range TT4 and TT3 concentrations at the time of surgery.. Furthermore, Group A cats had a significant reduction in TT3 concentrations over the treatment period whilst Group B cats did not suggesting this is the preferred treatment for clinical cases. As three cats developed liver pathology, biochemical monitoring of indices of hepatic damage should be undertaken regularly, particularly during the potassium iodate administration phase of the treatment regimen.

In summary, the use of propranolol and potassium iodate offer an alternative to presurgical treatment with carbimazole in cats that cannot tolerate this drug. The regimen used in group A (propranolol for 20 days and potassium iodate for the last 10 days) was both the most effective in lowering serum thyroid hormone concentrations and associated with the least adverse effects and this is protocol that we would recommend. Further studies are required to determine whether the drug combination has any additive or synergistic effects. Longer term studies would be required to assess the possibility of using these drugs for long term medical control and to see whether the cats' serum thyroid hormone concentrations would eventually 'escape' from the effects of the potassium iodate.

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Table 1

Summary Of Clinical Details Of The Cats Which Completed The Study

GROUP A CATS

CAT	AGE (years)	BREED	SEX	REASON FOR PRESENTATION	DOSE OF POTASSIUM IODATE (TID) DURING TRIAL (mg)	TT4 REFERENCE RANGE AT ANY TIME DURING TRIAL	TT4 REFERENCE RANGE AT TIME OF SURGERY
1	13	DSH	MN	Vaccination	42.50	No	No
2	14	DLH	FN	Vomiting	42.50	No	No
3	15	DSH	MN	Weight loss	42.50	No	No
4	14	DSH	FN	Weight loss	42.50	Yes	Yes
5	16	DSH	FN	Vaccination	21.25	No	No
6	9	DLH	FN	Weight loss	21.25	Yes	No
7	7	DSH	FN	Weight loss	21.25	No	No
8	8	DSH	FN	Polyphagia	21.25	No	No
9	8	DSH	FN	Heart murmur	21.25	Yes	Yes
10	12	DSH	MN	Weight loss	21.25	Yes	Yes
11	15	DSH	FN	Weight loss	21.25	Yes	Yes

GROUP B CATS

CAT	AGE (years)	BREED	SEX	REASON FOR PRESENTATION	DOSE OF POTASSIUM IODATE (TID) DURING TRIAL (mg)	TT4 REFERENCE RANGE AT ANY TIME DURING TRIAL	TT4 REFERENCE RANGE AT TIME OF SURGERY
12	15	DLH	MN	Vomiting	42.50	No	No
13	13	DSH	FN	PU/PD	42.50	No	No
14	10	DSH	FN	Weight loss	42.50	No	No
15	14	DSH	MN	Weight loss	42.50	Yes	No
16	14	DSH	FN	Polyphagia	21.25	No	No
17	10	DLH	FN	Weight loss	21.25	Yes	Yes
18	9	DSH	FN	Weight loss	21.25	Yes	No
19	15	DLH	MN	Vaccination	21.25	No	No
20	12	DSH	FN	Matted fur	21.25	No	No
21	12	DSH	FN	Anorexia	21.25	Yes	No

DLH - Domestic Long Hair
DSH - Domestic Short Hair
FN - Ovariohysterectomised female
MN - Castrated male
PD - Polydipsia
PU - Polyuria
TID- Three times daily
TT4 - Total serum thyroxine concentration

Table 2

Comparison Of Heart Rates Of Cats In Groups A and B Before, During And At The End Of Treatment Periods With Propranolol And Potassium Iodate

		Heart Rate (beats per minute)		
Cat Group	Day	Range	Median	Quartiles
A	1	180 - 280	240	220 260
	11	140 - 220	180	178 200
	20	140 - 200	180	160 180
B	1	180 - 270	250	195 260
	11	140 - 240	200	170 220
	20	140 - 210	180	175 200

Group A cats had propranolol from days 1 to 20 and potassium iodate from days 11 to 20 inclusive. Group B cats had potassium iodate from days 1 to 20 and propranolol from days 11 to 20 inclusive

Table 3

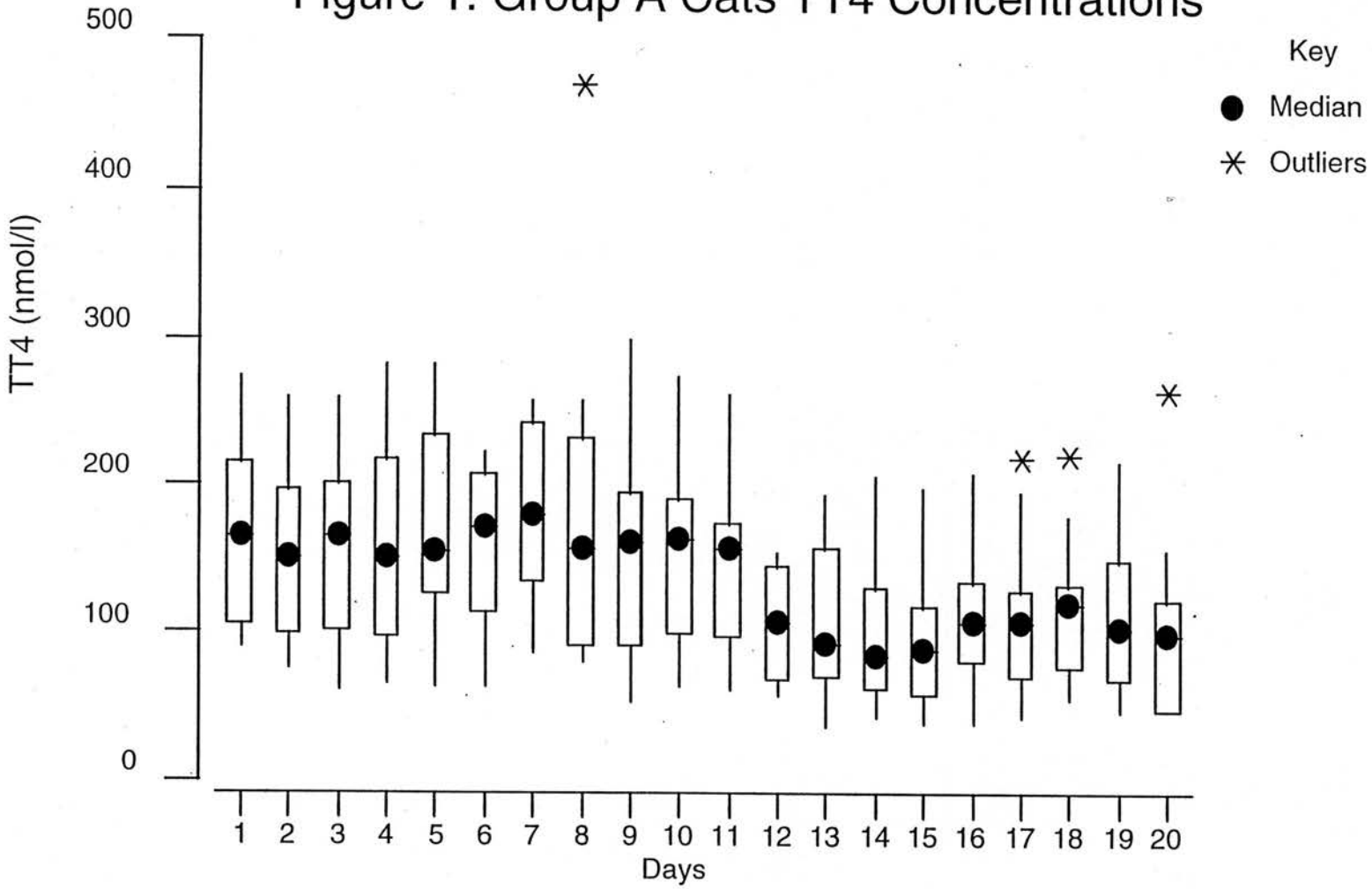
Comparison Of Serum Thyroid Hormone Concentrations Of Cats In Groups A And B Before, During And At the End Of The Treatment Periods With Propranolol And Potassium Iodate.

Thyroid Hormone	Cat Group	Day	Thyroid Hormone Concentration (nmol/l) Range	Median	Quartiles
		1	88.8 - 275.6	165.6	104.4 214.6
	A	11	62.5 - 275.8	155.7	95.4 173.2
		20	45.3 - 264.1	97.5	46.2 120.1
Thyroxine					
		1	88.6 - 313.2	219.7	105.7 285.3
	B	11	39.2 - 279.5	120.1	26.3 220.4
		20	39.5 - 453.5	120.1	84.0 212.9
—		1	3.16 - 5.26	4.06	3.48 4.37
	A	11	2.47 - 4.74	2.84	2.68 3.49
		20	1.18 - 2.60	1.76	1.36 2.38
Triiodothyronine					
		1	1.95 - 10.63	2.94	2.19 4.61
	B	11	0.54 - 5.87	1.91	0.83 3.05
		20	1.00 - 7.85	1.95	1.63 2.31
—		1	0.75 - 9.30	3.70	1.22 4.95
	A	11	0.62 - 5.55	3.05	1.21 4.36
		20	0.50 - 3.30	1.69	0.72 2.10
reverse Triiodothyronine					
		1	0.87 - 9.50	2.64	1.21 8.43
	B	11	0.54 - 4.90	1.72	0.86 3.88
		20	0.31 - 7.45	2.60	1.07 3.13

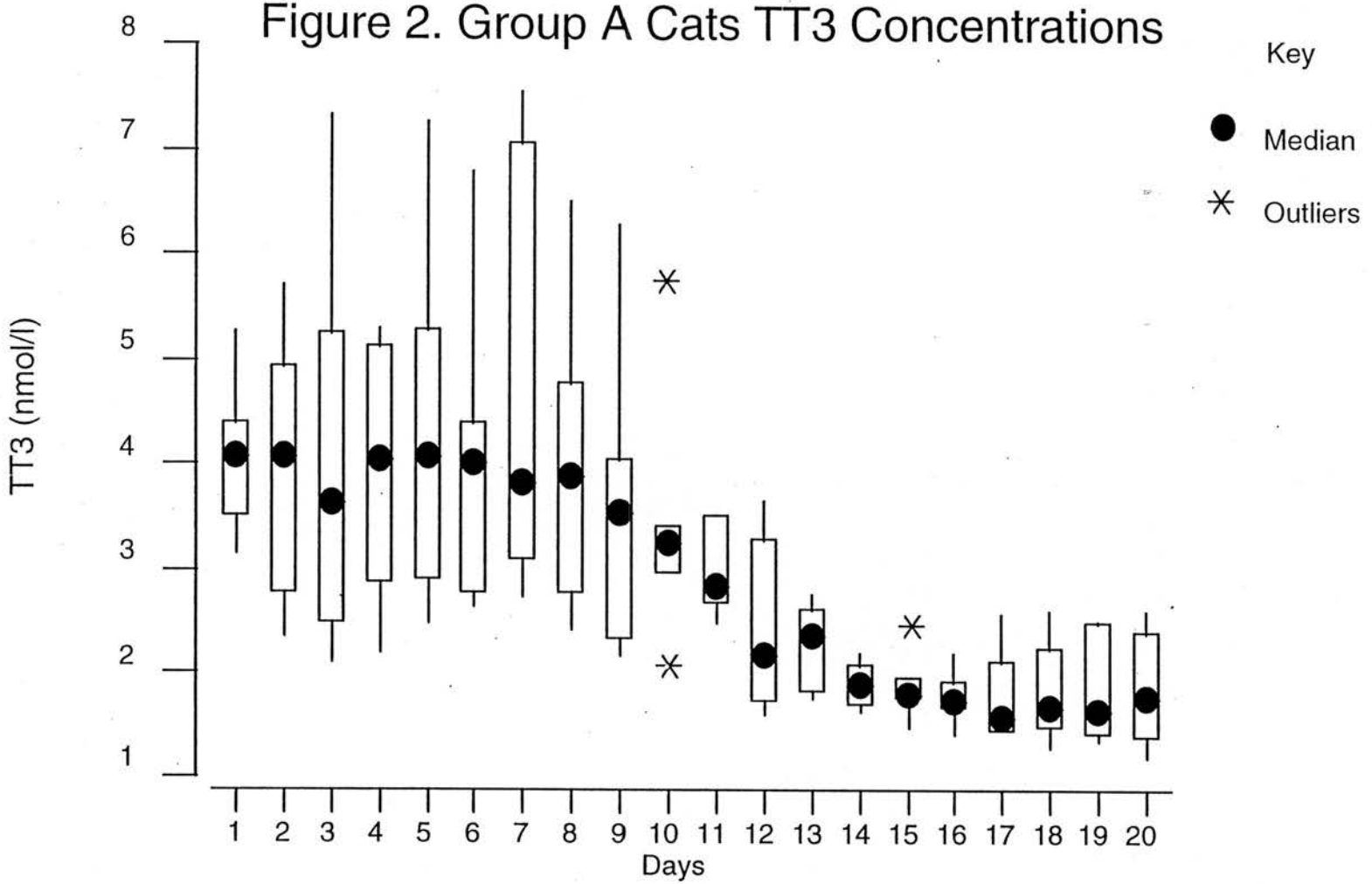
—

Group A cats had propranolol from days 1 to 20 and potassium iodate from days 11 to 20 inclusive. Group B cats had potassium iodate from days 1 to 20 and propranolol from days 11 to 20 inclusive

Figure 1. Group A Cats TT4 Concentrations

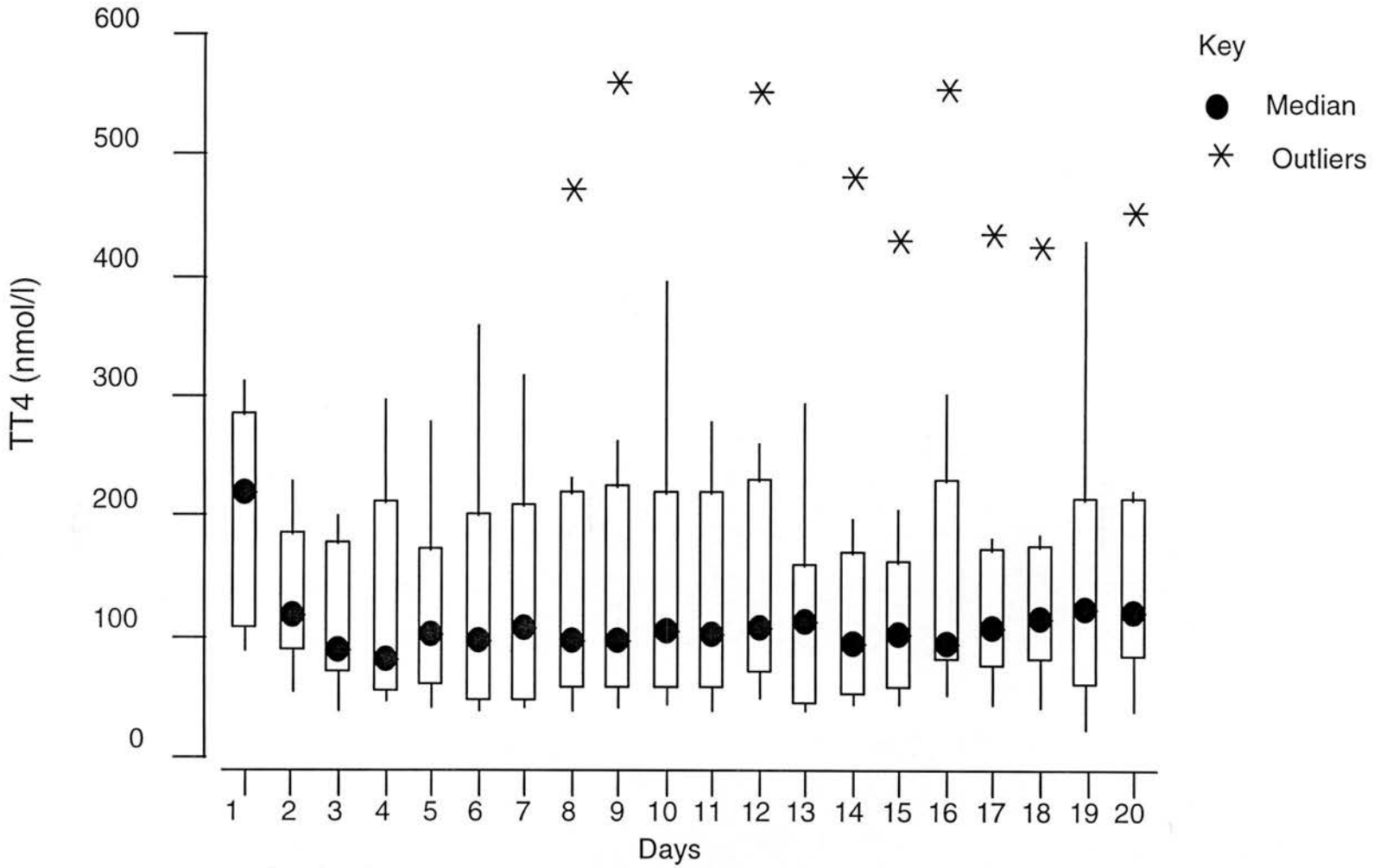


Boxplot of Group A cats showing serum total thyroxine (TT4) concentrations over the 20 day trial period. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3). Whiskers define the range of the data.



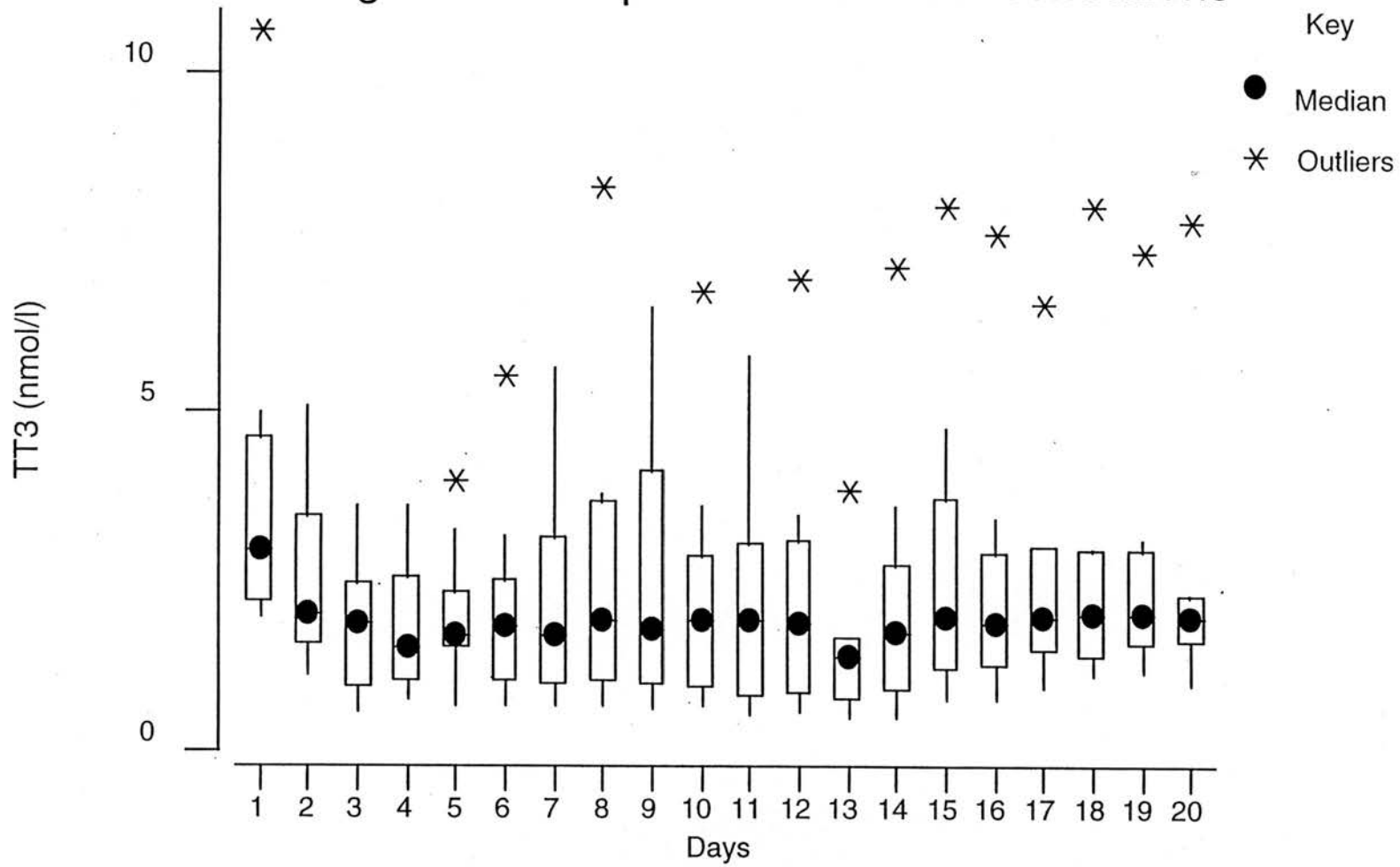
Boxplot of Group A cats showing total triiodothyronine (TT3) concentrations over the 20 day trial period. Bottom of the box represents the first quartile (Q1), the top of the box the third quartile (Q3). Whiskers define the range of the data.

Figure 3. Group B Cats TT4 Concentrations



Boxplot of Group B cats showing serum total T4 concentrations over the 20 day trial period. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3).
Whiskers define the range of the data.

Figure 4. Group B Cats TT3 Concentrations



Boxplot of Group B cats showing serum total T3 concentrations over the 20 day trial period. Bottom of the box represents the first quartile (Q1), the top of the box, the third quartile (Q3). Whiskers define the range of the data.