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Thyroid hormones and their effects: a new perspective

Abstract

The thyroid hormones are very hydrophobic and those that exhibit biological activity are 3',5',3,5-ltetraiodothyronine (T4), 3',5,3-l-triiodothyronine (T3), 3',5',3-l-triiodothyronine (rT3) and 3,5,-ldiiodothyronine (3,5-T2). At physiological pH, dissociation of the phenolic -OH group of these iodothyronines is an important determinant of their physical chemistry that impacts on their biological effects. When non-ionized these iodothyronines are strongly amphipathic. It is proposed that iodothyronines are normal constituents of biological membranes in vertebrates. In plasma of adult vertebrates, unbound T4 and T3 are regulated in the picomolar range whilst protein-bound T4 and T3 are maintained in the nanomolar range. The function of thyroid-hormone-binding plasma proteins is to ensure an even distribution throughout the body. Various iodothyronines are produced by three types of membrane-bound cellular deiodinase enzyme systems in vertebrates. The distribution of deiodinases varies between tissues and each has a distinct developmental profile. Thyroid hormones have many effects in vertebrates. It is proposed that there are several modes of action of these hormones. (1) The nuclear receptor mode is especially important in the thyroid hormone axis that controls plasma and cellular levels of these hormones. (2) These hormones are strongly associated with membranes in tissues and normally rigidify these membranes. (3) They also affect the acyl composition of membrane bilayers and it is suggested that this is due to the cells responding to thyroid-hormone-induced membrane rigidification. Both their immediate effects on the physical state of membranes and the consequent changes in membrane composition result in several other thyroid hormone effects. Effects on metabolism may be due primarily to membrane acyl changes. There are other actions of thyroid hormones involving membrane receptors and influences on cellular interactions with the extracellular matrix. The effects of thyroid hormones are reviewed and appear to be combinations of these various modes of action. During development, vertebrates show a surge in T4 and other thyroid hormones, as well as distinctive profiles in the appearance of the deiodinase enzymes and nuclear receptors. Evidence from the use of analogues supports multiple modes of action. Re-examination of data from the early 1960s supports a membrane action. Findings from receptor 'knockout' mice supports an important role for receptors in the development of the thyroid axis. These iodothyronines may be better thought of as 'vitamone'-like molecules than traditional hormonal messengers.

Disciplines

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Thyroid hormones and their effects: a new perspective

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ABSTRACT

The thyroid hormones are very hydrophobic and those that exhibit biological activity are 3′,5′,3,5-L-tetraiodothyronine (T4), 3′,5,3-L-triiodothyronine (T3), 3′,5′,3-L-triiodothyronine (rT3) and 3,5,-L-diiodothyronine (3,5-T2). At physiological pH, dissociation of the phenolic −OH group of these iodothyronines is an important determinant of their physical chemistry that impacts on their biological effects. When non-ionized these iodothyronines are strongly amphipathic. It is proposed that iodothyronines are normal constituents of biological membranes in vertebrates. In plasma of adult vertebrates, unbound T4 and T3 are regulated in the picomolar range whilst protein-bound T4 and T3 are maintained in the nanomolar range. The function of thyroid-hormone-binding plasma proteins is to ensure an even distribution throughout the body. Various iodothyronines are produced by three types of membrane-bound cellular deiodinase enzyme systems in vertebrates. The distribution of deiodinases varies between tissues and each has a distinct developmental profile. Thyroid hormones have many effects in vertebrates. It is proposed that there are several modes of action of these hormones. (1) The nuclear receptor mode is especially important in the thyroid hormone axis that controls plasma and cellular levels of these hormones. (2) These hormones are strongly associated with membranes in tissues and normally rigidify these membranes. (3) They also affect the acyl composition of membrane bilayers and it is suggested that this is due to the cells responding to thyroid-hormone-induced membrane rigidification. Both their immediate effects on the physical state of membranes and the consequent changes in membrane composition result in several other thyroid hormone effects. Effects on metabolism may be due primarily to membrane acyl changes. There are other actions of thyroid hormones involving membrane receptors and influences on cellular interactions with the extracellular matrix. The effects of thyroid hormones are reviewed and appear to be combinations of these various modes of action. During development, vertebrates show a surge in T4 and other thyroid hormones, as well as distinctive profiles in the appearance of the deiodinase enzymes and nuclear receptors. Evidence from the use of analogues supports multiple modes of action. Re-examination of data from the early 1960s supports a membrane action. Findings from receptor 'knockout' mice supports an important role for receptors in the development of the thyroid axis. These iodothyronines may be better thought of as 'vitamone'-like molecules than traditional hormonal messengers.

Key words: thyroxine, triiodothyronine, diiodothyronine, nuclear receptors, membranes, deiodinases, metabolism, growth, development, antioxidants.

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I. INTRODUCTION

On Christmas Day, 1914, thyroxine was first crystallised by Kendall. This was approximately 4500 years after the earliest recorded use of seaweed as an effective remedy for goitre, approximately a hundred years after the first isolation of elemental iodine, approximately forty years after the initial association of cretinism with the thyroid gland, twenty years after the demonstration by Magnus-Levy of the stimulatory effect of the thyroid on the metabolic rate of humans, and at approximately the same time as the discovery by Gudersnatch that tadpoles fed thyroid tissue precociously turn into frogs (Pitt-Rivers & Tata, 1959; Hetzel, 1989). Thyroxine is 3′,5′,3,5-L-tetra-iodothyronine and is nowadays commonly called T4.

The use of radioisotopes and paper chromatography in the 1940s led to the realisation that there were other important iodinated compounds apart from thyroxine. In Melbourne in 1948, an unknown iodothyronine was isolated using paper chromatography and suggested to be triiodothyronine (T3) (Hird & Trikojus, 1948). This was two years before the generally accepted first publication of the unknown iodinated thyronine (Gross *et al*., 1950) that was later isolated and identified as 3′,5,3-L-triiodothyronine and demonstrated to be more potent than T4 in preventing goitre (see Gross, 1993). Since the 1950s with the development of sophisticated analytical techniques we have realised that there are several other iodinated thyronines and their metabolites in the vertebrate body and that there exists a complex metabolic system involved with their interconversions.

Even before the discovery of T3 many effects of either an inactive or overactive thyroid gland were known and there was intense interest in the means whereby the thyroid gland exerted its hormonal effects on the body. Special attention was paid to its effects on metabolism as possibly being central to many of the other observed effects.

In the early 1960s, seminal work by Tata and colleagues shifted emphasis to protein synthesis. The finding of an early stimulation of ribonucleic acid synthesis (Tata & Widnell, 1966), followed by the discovery of specific nuclear T3 binding sites (Oppenheimer *et al*., 1972; Samuels & Tsai, 1973) led to the nuclear receptor hypothesis to explain thyroid hormone effects.

As an offshoot of the search for the genetic basis of cancer, in 1986 two groups reported that cellular equivalents of the viral erb-A gene coded for part of the thyroid hormone nuclear receptor. The genes for the two main types of thyroid nuclear receptor had been independently isolated from two different sources (Sap *et al*., 1986; Weinberger *et al*., 1986). These thyroid nuclear receptors are now recognised to be members of a superfamily of nuclear hormone receptors that bind to specific sections of the genome (see Jensen, 1991).

There are myriad effects of thyroid hormones that

have been recorded this century. A review by Hoch in 1962 on the biochemical actions of these hormones alone cited 611 references. Although much of the literature about the effects of thyroid hormones assumes that such effects are mediated by nuclear receptors, a more detailed search of the literature for the scientific basis of such statements turns up little direct evidence.

What is obvious from the literature is that a number of thyroid hormone effects are indubitably initiated by thyroid hormone receptors within the nucleus (e.g. thyroid-stimulating hormone (TSH) secretion in anterior pituitary, synthesis of malic enzyme in liver, growth hormone secretion), however, it is equally obvious that there are effects undoubtably non-genomic in origin (e.g. Ca^{2+} fluxes in mammalian erythrocytes, sugar uptake by thymocytes, action on heart membranes). What a search of the literature does suggest is that the sites of origin of the majority of thyroid hormone effects are still currently not known.

Following the discovery of T3, and the fact that nuclear receptors have a greater affinity for T3 than for T4, the belief developed that T3 is the active thyroid hormone and that T4 is only a 'prohormone'. The finding during the last decade that diiodothyronines (T2s) can also initiate thyroid hormone effects, and in some cases have a potency similar to that of T3, yet thyroid nuclear receptors have negligible affinity for T2, has also raised questions regarding the mode of action for thyroid hormone effects.

It is the purpose of this review to attempt a synthesis of what we currently know of thyroid hormones and their effects. The literature on the thyroid is vast and I am indebted to many excellent reviews that will be referenced in the appropriate sections. While much information is available for humans and the laboratory rat, I have not restricted myself to these two mammals but have taken a broader phylogenetic survey because of the power of an evolutionary perspective in understanding what is biologically important.

(1) Some prejudices

I come to this task with a background in the physiology of mammalian metabolism and its evolution. Many years ago, in youthful exuberance, I published a hypothesis that several thyroid hormone effects may be caused by changes in the fatty acid composition of membranes (Hulbert, 1978). Whilst my ideas have inevitably evolved since then, I will revisit this hypothesis, in order to examine if it still has some validity.

Another prejudice stems from my early physiological training. It is that the scientific use of the word 'regulation' involves homeostasis and therefore requires negative feedback to be involved somewhere. This meaning of 'regulation' is also applicable to biochemistry, with respect to the control of metabolism (see Fell, 1997). It is common to read statements like '…thyroid hormones regulate…(a particular enzyme, activity etc.)'. However, in general, negative feedback from thyroid hormone effects to the thyroid are not evident. For example, using this rather strict physiological definition, the thyroid hormones do not 'regulate' metabolism. If they did, a decreased metabolic rate should result either in increased hormonal secretion or increased hormonal effect (or both) in order to 'regulate' metabolic rate. An opposite response is expected for an increased metabolic rate. The objection to the improper use of the word 'regulate' is, I believe, more than a pedantic quibble. It is important in that it influences how we think about what these hormones are doing. Indeed, often a more appropriate phrasing is that thyroid hormones 'influence' (or stimulate, inhibit, initiate) a certain enzyme, activity, developmental event etc.

One exception to the general lack of negative feedback between thyroid hormone effects and the thyroid is, of course, TSH release from the anterior pituitary. With respect to the thyroid, what appears to be 'regulated' are the levels of thyroid hormones themselves. This is also true when we examine the cellular level of organization. The thyroid hormones are metabolised within cells *via* various deiodination mechanisms and in some tissues (most notably the brain), there is evidence of negative feedback between thyroid status and the deiodination systems. The consequence of this feedback is that both the whole organism and some individual cells attempt to maintain a relatively constant concentration of thyroid hormone despite varying conditions. Viewed in this light, the thyroid hormones are more akin to an important body constituent, a 'vitamin-like' molecule rather than a classical 'hormonal messenger'.

Such a perspective has been raised, in the context of the wide phylogenetic distribution of these molecules, in an interesting recent review by Eales (1997) where he suggested the term 'vitamone' to describe such a role. In this light, it is also of interest that the superfamily to which thyroid nuclear receptors belong includes those for vitamin D and

(vitamin-A-related) retinoic acid (Jensen, 1991). This perspective suggests that due to the relative constancy of their concentration, thyroid hormones are probably best regarded not as 'regulatory' hormones in the normal sense but as 'permissive' hormones, possibly 'vitamones'.

II. THYROID HORMONES

(1) Structure and physical chemistry

Thyroid tissue consisting of follicles of epithelial cells surrounding a lumen filled with colloid has been identified in all vertebrates examined to date. In most vertebrates, these follicles are grouped together into a discrete gland, the thyroid, whilst in others they are diffusely distributed generally in the anterior region of the body (Etkin & Gona, 1974). This review will restrict itself to vertebrates. For two fascinating recent accounts of the function of thyroid hormones in invertebrates the reader is referred to Eales (1997) and Johnson (1997).

The thyroid epithelial cells take up iodide and secrete thyroglobulin protein into the central lumen of the follicle. Of the more than 100 tyrosine residues per molecule of thyroglobulin, a number are iodinated *ortho* to the phenolic hydroxyl group to form 3-iodotyrosine and 3,5-diiodotyrosine. The spatial arrangement of thyroglobulin favours the coupling of some of these iodotyrosines to form T4 which is secreted from the thyroid follicle. In cases of iodine deficiency, relatively more T3 and less T4 is synthesised and secreted. This review will not concern itself with events in the thyroid follicle but will concentrate on the thyroid hormones themselves and how they exert their multitudinous effects. In view of the relative ease of synthesis of these molecules (e.g. both T4 and T3 can be synthesised by non-enzymatic means), their wide phylogenetic distribution is not surprising (see Eales, 1997).

The chemical structure of the thyroid hormones T4 and T3 is illustrated in Fig. 1 together with a molecular model showing the outer phenolic ring oriented in a plane perpendicular to the inner ring. Also included in Fig. 1 are another two iodothyronines, reverse T3 (rT3) and 3,5,-diiodothyronine, that exert some thyroid hormonal effects and will be discussed in later sections. Because they are synthesised from natural tyrosine residues, the amino acid part of the molecule is of the L-isomer configuration. The molecular mass of T4 and T3 is 777 and 651 Da, respectively. The thyroid hormones (and their metabolites) are the only known molecules in the body that contain iodine and most of the molecular mass of both thyroid hormones is contributed by their iodine atoms which are, by far, the

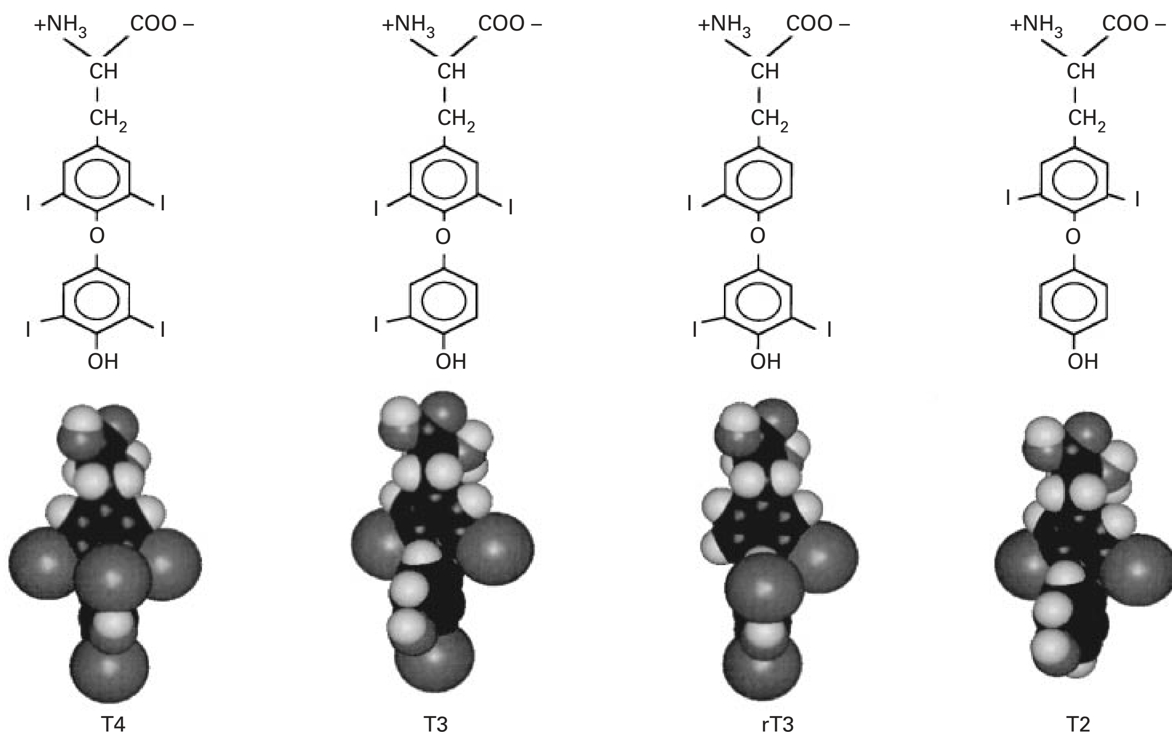

Fig. 1. The chemical structures and molecular models of four biologically active thyroid hormones; thyroxine (T4), 3′,3,5-triiodothyronine (T3), 3′,5′,3-triiodothyronine (rT3) and 3,5-diiodothyronine (T2).

heaviest elements found in the body (atomic mass 127). The next heaviest element found in the body is selenium (atomic mass 79) which is an important part of the enzymes that deiodinate the thyroid hormones. The iodine atoms also have strong electron binding energies and are very electron attractive.

The amino group, carboxyl group and phenolic −OH group are all ionizable parts of these molecules. The relative ionization of the phenolic group in different thyronines will be a major influence on their relative lipophilicity. Simple phenolic groups are 50% dissociated at pH of approximately 10 (i.e they have a pK of approximately 10). The presence of a single iodine (with its electron attraction) on the outer ring of T3 reduces the pK of its phenolic group to 8.45, whilst the two iodine atoms on the outer ring of T4 further reduces the pK of its phenolic group to 6.73 (Gemmill, 1955). At physiological pH approximately 80% of T4 molecules have the phenolic hydroxyl group in the ionized form whilst only approximately 10% of T3 molecules have an ionized phenolic hydroxyl group (Korcek & Tabachnick, 1976). This difference will result in different relative hydrophobicities of T4 and T3 at physiological pH.

Both molecules are hydrophobic molecules, with T3 being more so than T4 because of its relative lack of an ionized phenolic group. They both have a low solubility at neutral and acid pHs but dramatically increased solubility in alkaline solutions, because of the increased ionization of the phenolic end of the molecule. For example, the solubility limit of T4 in aqueous solution rises from 2.3 μM at pH 7, to 4.5 μM at pH 9, and to 260 μM at pH 11 (values taken from Schreiber & Richardson, 1997). This is the basis for the common practice of using alkaline solutions for injection of thyroid hormones. Whilst the hormones are soluble in the injectate, once inside the body they will return to a more neutral (or even acidic) environment and thus will be more hydrophobic in character. Hillier (1970) reported that at physiological pH the 'partition coefficient' between phospholipid (phosphatidylcholine) and the aqueous environment was 12000 for T4 and 22000 for T3. Dickson *et al*. (1987) report values of 17500 and 23500 for T4 and T3 respectively.

(2) **Plasma concentrations and distribution to the tissues**

Whilst circulating T4 originates from the thyroid follicles, circulating T3 comes predominantly from the deiodination of T4, both in the thyroid itself and in peripheral tissues. This deiodination will be discussed in Section II.3. Other iodothyronines are also formed by the deiodination mechanisms and these are present in small concentrations in the plasma. Both T4 and T3 are found in the plasma either bound to plasma proteins or in the free (unbound) state. Information concerning both the total and free concentration of T4 and T3 in plasma from a diverse range of vertebrates under a number of conditions is collated in Table 1. Much of this information has been obtained from the values for controls in particular experiments, some have been interpolated from graphs, and many values have been recalculated in SI units. Selected values for earlier stages in the life cycle of some species are also included. Fig. 2 presents plasma T4 and T3 concentrations for normal (= euthyroid) adult vertebrates relative to their body mass. Many of the reports cited in Table 1 did not report body mass of the animals examined. For these I have estimated the adult body mass of the particular species. Table 1 and Fig. 2 also include the ranges regarded as typically 'euthyroid' for humans (from Stockigt, 1996).

In normal adult vertebrates, the plasma total concentration of T4 is in the nanomolar range with an average of approximately 35 nM and ranges from below measurable levels in some amphibians to 148 nM in the hedgehog, *Erinaceus europaeus*. The plasma total concentration of T3 is also in the nanomolar range for vertebrates and averages approximately 3.5 nM, ranging from unmeasurable to a maximum of 76 nM reported for the parrotfish, *Sparisoma* spp. The vast majority of both T4 and T3 is bound to plasma proteins with the free hormone being in the picomolar range for all vertebrates, approximating 0.01–0.1% of the total hormone concentration. For adult vertebrates, the average free T4 and free T3 plasma concentrations are approximately 40–50 pM, with respective ranges of 2–600 pM and 1–492 pM. It is the level of 'free' thyroid hormones that is the more physiologically important concentration (Robbins & Rall, 1957; Ekins, 1986; Mendel, 1989).

In many cases reported in Table 1, the concentrations in the early phase of the life cycle are considerably higher than those observed in the adult phase. This is especially true for periods of 'metamorphic' change in some of the vertebrates. This developmental surge of thyroid hormones will be discussed later. Although the hormone concentrations are presented as a single value for each species, of course the normal euthyroid hormone

Table 1. *Concentrations of thyroxine (T4) & triiodothyronine (T3) in vertebrate plasma*

Vertebrate	*Species*	Condition	Body mass (g)	Total [T4] (nM)	Total [T3] (nM)	Free [T4] (pM)	Free [T3] (pM)	Reference
Protochordate								
Ascidean	*Phallusia mammillata*	Adults		0.3	0.0			Fredriksson *et al.* (1993)
Agnathan								
Sea lamprey	*Petromyzon marinus*	Ammocoete larvae		65.0	24.0	282.0	116.0	Weirich *et al.* (1987)
Sea lamprey	*Petromyzon marinus*	Juvenile	50	4.6	0.4			Youson *et al.* (1994)
Sea lamprey	*Petromyzon marinus*	Spawning adult		12.0	1.6	25.2	1.1	Weirich *et al.* (1987)
Sea lamprey	*Petromyzon marinus*	Spawning female		104.0	0.7			Hornsey (1977)
Sea lamprey	*Petromyzon marinus*	Adult males		12.0	1.7			Hornsey (1977)
Sea lamprey	*Petromyzon marinus*	Adults	200	3.5	3.0			Sower *et al.* (1985)
Fish								
Salmon	*Oncorhynchus keta*	Eggs		19.0				Tagawa & Hirano (1987)
Salmon	Various species	Embryos		9–19	1.5–7.7			Sullivan *et al.* (1987)
Masu salmon	*Oncorhynchus masou*	Peak smolt	19	8.4	1.2			Ura *et al.* (1994)
Salmon	*Oncorhynchus kisutch*	Pre-smolt	10	15.0	9.2			Swanson & Dickhoff (1987)
Salmon	*Oncorhynchus kisutch*	Transition		31.0	7.6			Swanson & Dickhoff (1987)
Salmon	*Oncorhynchus kisutch*	Smolt	30	15.0	4.6			Swanson & Dickhoff (1987)
Salmon	*Oncorhynchus kisutch*	Wild smolt		4.5	4.6			Whitesel (1992)
Salmon	*Oncorhynchus kisutch*	Wild parr		2.3	3.5			Whitesel (1992)
Rainbow trout	*Oncorhynchus mykiss*	Immature	110	4.0	14.0			Gelineau *et al.* (1996)
Rainbow trout	*Oncorhynchus mykiss*	Immature	55	9.5	7.4			Reddy & Leatherland (1995)
Rainbow trout	*Oncorhynchus mykiss*	Immature	100	12.6	10.2			Holloway *et al.* (1994)
Rainbow trout	*Oncorhynchus mykiss*	Adult		1–6	2–4			Eales *et al.* (1981)
Arctic charr	*Salvelinus alpinus*		206	2.7	2.4	5.0	3.2	Eales & Shostak (1985)
Brown trout	*Salmo trutta*	Young adults	400	1.9	0.9			Waring & Brown (1997)
Atlantic cod	*Gadus morhua*	Male adults	750	3.6	19.3			Cyr *et al.* (1998)
Rainbow trout	*Oncorhynchus mykiss*	Adults	1150	7.1	3.3			Gomez *et al.* (1997)
Lake trout	*Salvelinus namaycush*	Adults		10.0	3.4	9.9	3.2	Weirich *et al.* (1987)
Baltic salmon	*Salmo salar*	Adults		11.0				Larsson *et al.* (1985)

Goldfish	*Carassius auratus*	Adults	20–35	1.0	3.4			MacKenzie *et al*. (1987)
Squirrelfish	*Holocentrus rufus*	Adults	91	34.1	14.4	599.7	491.6	Eales & Shostak (1987)
Coney	*Cephalopholis fulva*	Adults	237	4.4	4.0	30.9	24.6	Eales & Shostak (1987)
Grunt	*Haemulon flavolineatum*	Adults	65	13.0	10.4	70.8	46.1	Eales & Shostak (1987)
Banded butterflyfish	*Chaetodon striatus*	Adults	72	14.0	5.7	64.4	33.8	Eales & Shostak (1987)
Rock beauty	*Holacanthus tricolor*	Adults	98	5.4	6.3	23.2	24.6	Eales & Shostak (1987)
Parrotfish	*Sparisoma* spp.	Adults	120	54.3	76.2	303.7	419.4	Eales & Shostak (1987)
Blue tang	*Acanthurus coeruleus*	Adults	104	5.0	4.6	23.2	39.9	Eales & Shostak (1987)
Ocean surgeonfish	*Acanthurus bahianus*	Adults	74	4.8	2.3	48.9	21.5	Eales & Shostak (1987)
Orange-spotted filefish	*Cantherines pullus*	Adults	69	1.9	0.3	2.1	0.6	Eales & Shostak (1987)
Spotted goatfish	*Pseudupeneus maculatus*	Adults	137	17.6	5.4	100.4	24.6	Eales & Shostak (1987)
Australian lungfish	*Neoceratodus forsteri*	Adults		6.5	0.4			J. M. P. Joss personal comm.
Amphibians								
Salamander	*Eurycea bislineata*	Peak-metamorphic		10.0	1.6			Alberch *et al*. (1986)
Salamander	*Eurycea bislineata*	Adult		0.0	0.0			Alberch *et al*. (1986)
Frog	*Rana clamitans*	Peak-metamorphic		9.0				Weil (1986)
Bullfrog	*Rana catesbiana*	Peak-metamorphic		6.0	2.2			Suzuki & Suzuki (1981)
Bullfrog	*Rana catesbiana*	Adults		0.5	0.3			Suzuki & Suzuki (1981)
Frog	*Bufo regularis*	Adults		3.0				Larsson *et al*. (1985)
Frog	*Rana ridibinda*	Adults		0.1–2				Vandorpe *et al*. (1987)
Toad	*Bufo japonicus*	Female adults		0.05–1	0–0.2			Tasaki *et al*. (1986)
Reptiles								
Lizard	*Sceloporus undulatus*	Yearlings	7.7	5.5				Gerwein & John-Alder (1992)
Lizard	*Sceloporus undulatus*	Captive adults	10.7	11.1	3.1			John-Alder & Joos (1991)
Lizard	*Sceloporus undulatus*	Field adults	8.3	13.1	3.2			John-Alder & Joos (1991)
Lizard	*Ameiva undulata*	Captive	23	8.2				Steinberg *et al*. (1993)
Lizard	*Dipsosaurus dorsalis*	Adult males	58	14.5				John-Alder (1984a)
Lizard	*Dipsosaurus dorsalis*	Adult males	56	3.2				John-Alder (1983)
Lizard	*Dipsosaurus dorsalis*	Hibernating adult		1.3				John-Alder (1984b)
Lizard	*Dipsosaurus dorsalis*	Adult (spring)		13.0	0.5			John-Alder (1984b)

Table 1 (*cont.*)

Vertebrate	*Species*	Condition	Body mass (g)	Total [T4] (nM)	Total [T3] (nM)	Free [T4] (pM)	Free [T3] (pM)	Reference
Reptiles (*cont.*)								
Turtle	*Trachemys scripta*	Growing (expt 1)	80	145.0				Denver & Licht (1991)
Turtle	*Trachemys scripta*	Growing (expt 2)	80	83.7				Denver & Licht (1991)
Garter snake	*Thamnophis sirtalis*	Adults	49.5	1.3	0.3			Etheridge (1993)
Viper	*Viper aspis*	Adult hibernating	100	3.4				Naulleau *et al.* (1987)
Viper	*Viper aspis*	Adult spring	85	12.7				Naulleau *et al.* (1987)
Green sea turtle	*Chelonia mydas*	Adult		12.5				Licht *et al.* (1985)
Lizard	*Trachydosaurus rugosus*	Adult	520	3.0	0.3			Hulbert & Williams (1988)
Tortoise	*Chelodina longicollis*	Adult	800	1.0	0.3			Hulbert & Williams (1988)
Crocodile	*Crocodylus johnstonii*	Adult	1150	3.0	0.5			Hulbert & Williams (1988)
Lizard	*Podarcis sicula*	Adult	9		0.15		1.7	Venditti *et al.* (1996)
Birds								
Japanese quail	*Coturnix japonica*	Hatchling		38.6	5.1			McNabb & Olson (1996)
Japanese quail	*Coturnix japonica*	Adult		21.0	2.5	18.5	12.5	McNabb *et al.* (1984a)
Ring dove	*Streptopelia risoria*	Adult		23.0	6.8	8.7	4.6	McNabb *et al.* (1984a)
Ring dove	*Streptopelia risoria*	Hatchling		5.0	0.8			McNabb & Cheng (1985)
Ring dove	*Streptopelia risoria*	Adult		22.0	6.9			McNabb & Cheng (1985)
Ring dove	*Streptopelia risoria*	10 days post hatching		18.0	5.1			McNabb & Olson (1996)
Starling	*Sturnus vulgaris*	10 days post hatching		15.4	2.6			McNabb & Olson (1996)
Redwing blackbird	*Agelaius phoenicus*	8 days post hatching		23.2	3.9			McNabb & Olson (1996)
Emperor penguin	*Aptenodytes forsteri*	Adult	23000	15.9	1.6			Groscolas & Leloup (1989)
Chicken	*Gallus domesticus*	Egg		10.0	3.1			Sechman & Bobek (1988)
Chicken	*Gallus domesticus*	Immature		12.0	9.2			Williamson & Davison (1987)

Chicken	*Gallus domesticus*	Adult		22.0				Larsson *et al*. (1985)
Chicken	*Gallus domesticus*	Adult		22.0	1.6			Rudas & Pethes (1986)
Chicken	*Gallus domesticus*	Adult	4300	27.0	1.3			Bruggeman *et al*. (1997)
Chicken	*Gallus domesticus*	Adult (30 day)	731		1.5		9.5	Di Meo *et al*. (1993)
Pigeon	*Columbia livia*	Adult females	400	34.2	2.5			John *et al*. (1995)
Ostrich	*Struthio camelus*	Hatchling	870	7.6				Dawson *et al*. (1996)
Ostrich	*Struthio camelus*	Adult	120 000	1.8				Dawson *et al*. (1996)
Mammals								
Platypus	*Ornithorhynchus anatinus*	Adult	1070	60.3	0.9			Hulbert & Grant (1983)
Platypus	*Ornithorhynchus anatinus*	Juvenile	760	107.5	1.5			Hulbert & Grant (1983)
Echidna	*Tachyglossus aculeatus*	Adult	3000	15.2	1.6	20.3	4.5	Nicol *et al*. (2000)
Echidna	*Tachyglossus aculeatus*	Adult	3000	15.7	0.7			Hulbert & Augee (1982)
Bandicoot	*Isoodon macrourus*	Adult	1500	22.0	1.5			Hulbert & Augee (1982)
Wallaby	*Macropus eugenii*	Adult	6000	12.2	0.8	8.1	41.9	Janssens *et al*. (1990)
Wallaby	*Macropus eugenii*	Pouch-young (peak)		81.2	3.1	44.9	166.0	Janssens *et al*. (1990)
Koala	*Phascolarctus cinereus*	Adults	9000	3.2	0.4	3.3	1.4	Lawson *et al*. (1996)
Shrew	*Sorex vagrans*	Adults	5.2	17.4				Tomasi (1984)
Rodent	*Peromyscus maniculatus*	Adults	18.4	45.7				Tomasi (1984)
Rodent	*Reithrodontomys megalotis*	Adults	12.3	30.9				Tomasi (1984)
Rodent	*Microtus montanus*	Adults	29	43.6				Tomasi (1984)
Brandt's vole	*Microtus brandtii*	Adults	33	45.0	1.7			Liu *et al*. (1997)
Mouse	*Mus musculus*	Adults		61.0	1.1			Burgi *et al*. (1986)
Mouse	*Mus musculus*	Severe infection		21.0	0.4			Burgi *et al*. (1986)
Mouse	*Mus musculus*	Adult		39.7	1.3	14.1	7.6	Palha *et al*. (1994)
Mouse	*Mus musculus*	TTR null adult		18.9	1.1	15.7	7.6	Palha *et al*. (1994)
Hamster	*Mesocricetus auratus*	Adult males	146	46.3	0.7			Tomasi & Horwitz (1987)
Hedgehog	*Erinaceus europaeus*	Active male	700	148.0				Fowler (1988)
Hedgehog	*Erinaceus europaeus*	Active female	900	103.0				Fowler (1988)
Rat	*Rattus norvegicus*	Adult male	283	65.6	0.8			Ribeiro *et al*. (1997)
Rat	*Rattus norvegicus*	MSG obese	224	51.5	0.9			Ribeiro *et al*. (1997)
Rat	*Rattus norvegicus*	Adult	200–300	43.8	0.9	16.7	1.7	Woody *et al*. (1998)
Rat	*Rattus norvegicus*	Exercised adult	200–300	45.0	0.9	15.4	2.3	Woody *et al*. (1998)

Table 1 (*cont.*)

Vertebrate	*Species*	Condition	Body mass (g)	Total [T4] (nM)	Total [T3] (nM)	Free [T4] (pM)	Free [T3] (pM)	Reference
Mammals (*cont.*)								
Rat	*Rattus norvegicus*	Adult (young)		63.0	1.3	36.0	6.0	Chen & Walfish (1978)
Rat	*Rattus norvegicus*	Adult (old)		30.0	1.2	15.0	5.0	Chen & Walfish (1978)
Rat	*Rattus norvegicus*	Adult		80.0	1.4	67.0	16.0	Weirich *et al.* (1987)
Rat	*Rattus norvegicus*	Fetal (22 days)		6.0	0.2			Ruiz de Ona *et al.* (1988)
Rat	*Rattus norvegicus*	Adult (PUFA diet)	400	68.0	0.5			Takeuchi *et al.* (1995)
Rat	*Rattus norvegicus*	Adult (SFA diet)	400	65.0	0.4			Takeuchi *et al.* (1995)
Guinea pig	*Cavia porcellus*	Fetal (62 days)		49.9	0.3	66.0	1.8	Castro *et al.* (1986)
Guinea pig	*Cavia porcellus*	Adult		39.0	0.6	17.0	4.0	Castro *et al.* (1986)
Cat	*Felis domesticus*	Healthy adults		26.0		24.8		Mooney *et al.* (1996)
Cat	*Felis domesticus*	Healthy adults				15.0		Kyle *et al.* (1994)
Cat	*Felis domesticus*	Adult		36.0				Larsson *et al.* (1985)
Cat	*Felis domesticus*	Adult		34.6		22.9		Paradis & Page (1996)
Rabbit	*Oryctolagus cuniculus*	Adult		22.0				Larsson *et al.* (1985)
Woodchuck	*Marmota monax*	Adult (fall)	3900			2.7		Rawson *et al.* (1998)
Woodchuck	*Marmota monax*	Adult (spring)	3600			8.4		Rawson *et al.* (1998)
Dog	*Canis familiaris*	Adult		29.0				Larsson *et al.* (1985)
Dog	*Canis familiaris*	Adult	32 600	28.0	1.5	22.0		Peterson *et al.* (1997)
Dog	*Canis familiaris*	Adult		40.0		19.6		Paradis *et al.* (1996)
Dog	*Canis familiaris*	Adult		25.0	1.1			Miller *et al.* (1992)
Dog	*Canis familiaris*	Adult	13 800	48.8	0.4			Minten *et al.* (1985)
Monkey	*Macaca mulatta*	Adult		56.0				Larsson *et al.* (1985)
Lamb	*Ovis familiaris*	Fetal		100.0	3.0	30.0	2.1	Wrutniak *et al.* (1985)
Sheep	*Ovis familiaris*	Adult		83.0				Larsson *et al.* (1985)
Nubian goat	*	Newborn	4200	64.4	235.5			De la Colina *et al.* (1993)
Nubian goat	*	21 days old	5600	86.2	103.0			De la Colina *et al.* (1993)
Goat	*Capra hircus*	Adult		94.0				Larsson *et al.* (1985)
Goat	*Capra hircus*	Adult		83.7	3.7			Colavita *et al.* (1983)
Pig	*Sus scrofa*	Adult		46.0				Larsson *et al.* (1985)
Black bear	*Ursus americanus*	Adult	91 000	25.7	1.0	5.1	2.6	Tomasi *et al.* (1998)
Harbour seal	*Phoca vitulina*	Mixed				9.8	2.9	Schumacher *et al.* (1995)
Gray seal	*Halichoerus grypus*	Adult	90 000	29.9	0.6	14.0		Boily (1996)

Weddell seal	*Leptonychotes weddelli*	Adult		9.3	1.5			Schumacher *et al*. (1992)
Cattle	*Bos taurus*	Adult		49.0				Larsson *et al*. (1985)
Cattle	*Bos taurus*	Adult (non-lactating)		64.3	1.7			Tiirats (1997)
Cattle	*Bos taurus*	Adult (lactating)		45.1	1.7			Tiirats (1997)
Horse	*Equus domesticus*	At rest		8.6	0.7			Gonzalez *et al*. (1998)
Horse	*Equus domesticus*	After exercise		9.0	1.0			Gonzalez *et al*. (1998)
Horse	*Equus domesticus*	Adults	525 000	19.9	1.0	11.6	2.1	Messer *et al*. (1995)
Horse	*Equus domesticus*	Adults		34.8	1.2	33.2	8.8	Irvine & Evans (1975)
New world primates	*Mixed species*	Adults		38.0	2.6	31.0		Arbelle *et al*. (1994)
Old world primates	*Mixed species*	Adults		71.0	2.8	34.0		Arbelle *et al*. (1994)
Human	*Homo sapiens*	Adults		91.6	1.9	16.4	6.4	Franklyn *et al*. (1985)
Human	*Homo sapiens*	Adults		115.0	2.2			Wrutniak *et al*. (1985)
Human	*Homo sapiens*	Adults			1.8	14.3		Maes *et al*. (1997)
Human	*Homo sapiens*	Adult (pre antarctic)	78 000	94.0	2.2	32.2	5.0	Reed *et al*. (1986)
Human	*Homo sapiens*	Adult (post antarctic)	79 000	88.8	2.0	29.6	4.1	Reed *et al*. (1986)
Human	*Homo sapiens*	Euthyroid range		60–140	1–3	10–25	3–8	Stockigt (1996)

TTR, transthyretin; MSG, monosodium glutamate; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acids; *specific name of Nubian goat was not given in the source reference.

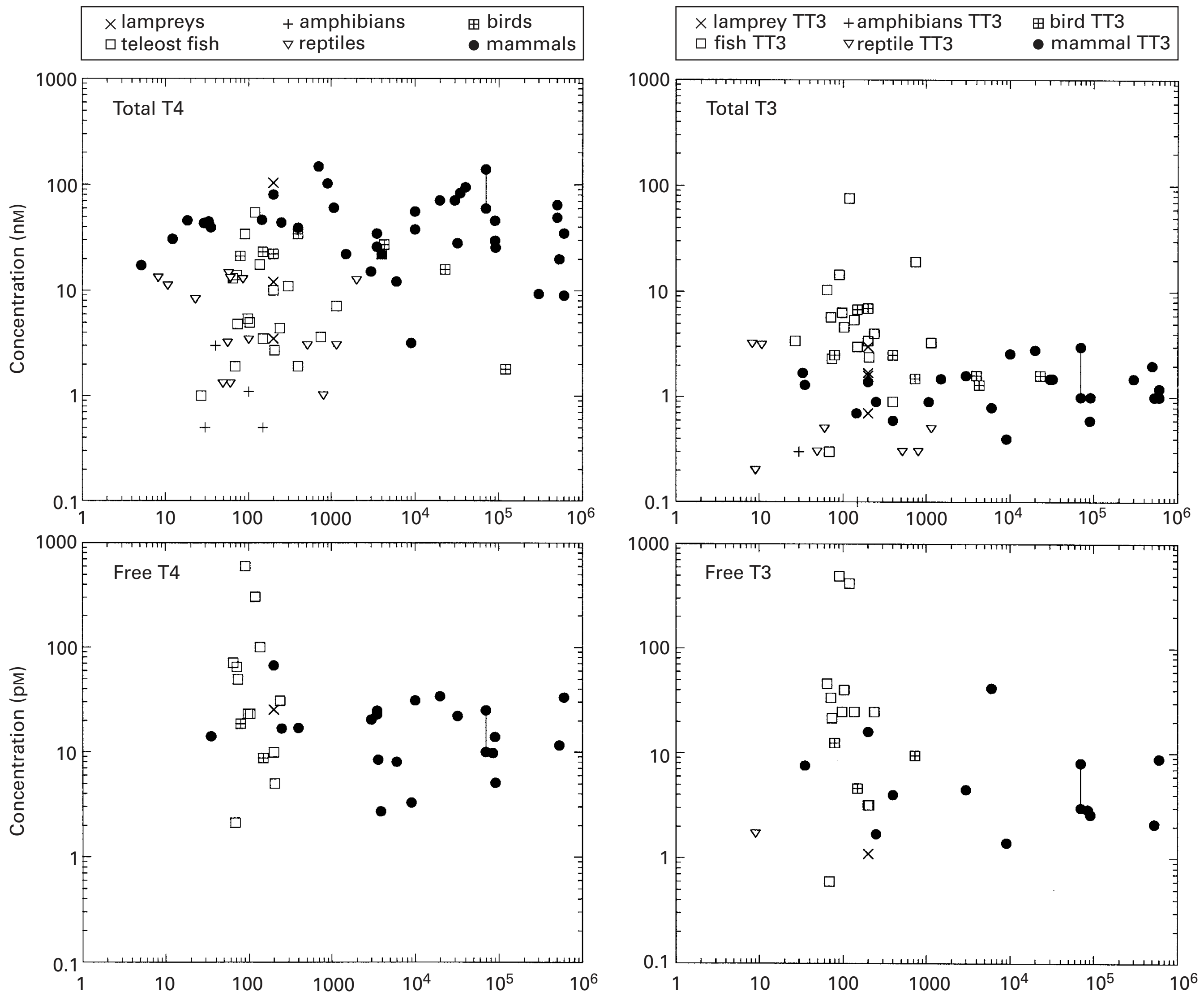

Fig. 2. The concentrations of total and free thyroid hormones (T4 and T3) in the plasma of adult vertebrates. The ranges regarded as euthyroid in humans are represented by a vertical line in each graph. All data are from Table 1.

concentrations are regarded as a range. For humans the approximate normal euthyroid range is typically regarded as 60–140 nM for total T4, 1–3 nM for total T3, 10–25 pM for free T4 and 3–8 pM for free T3 (taken from Stockigt, 1996). The free hormone values especially are influenced by measurement techniques. As can be seen from Fig. 2, the values for fish span the entire range observed in vertebrates. This is the case for total and free levels of both T4 and T3. The fact that most of these values come from a single study (Eales & Shostak, 1987) shows that this is a real situation and not an artifact of interlaboratory variation.

Although there are only a few values for some vertebrate classes, it appears from Fig. 2 that there is no consistent pattern of different free hormone levels in different vertebrate classes. This is not the case for the total thyroid hormone levels where there seem to be patterns for different classes of vertebrates. For example, apart from fish, there seems to be a trend of increasing total T4 going from amphibians to reptiles, to birds, to mammals. For total T3, the levels tend to increase from amphibians to reptiles, to mammals, to birds. The total levels of hormone will be greatly influenced by a combination of the plasma concentrations of thyroid-hormone-binding proteins together with their relative affinity for T4 and T3. In this light, it is of interest that one of the main plasma binding proteins in higher vertebrates, transthyretin, has recently been shown to have a higher affinity for T3 than T4 in birds, but higher affinity for T4 than T3 in mammals (Chang *et al.*,

1998). Thus, total thyroid hormone levels in plasma are likely to be largely a reflection of thyroid-hormone-binding plasma proteins. They do not necessarily indicate that one species of vertebrate is more hyperthyroid than another.

Another factor of note from Fig. 2 is that there is no relationship between body mass and the plasma concentration of thyroid hormones. The lack of a correlation between body mass of mammals and either free T4 or free T3 levels is of interest in view of the well-known influence of body mass on basal metabolism. On a body-mass basis, mice have a metabolic rate approximately 20-fold greater than large mammals such as horses and cattle (Kleiber, 1961) yet the free thyroid hormone levels in their plasma are similar. In the same manner, free thyroid hormone concentrations are not greatly different between ectothermic and endothermic vertebrates although the resting metabolism of an endotherm is 5–10 times that of a similar-sized ectothermic vertebrate at the same body temperature (Hulbert, 1980*b*).

The binding of T4 and T3 to plasma proteins has, for obvious reasons, been most studied in humans where three main thyroid-hormone-binding proteins have been identified. These are thyroxine-binding globulin (TBG), transthyretin (TTR, previously known as thyroxine-binding prealbumin) and albumin (ALB). Some T4 is also bound to plasma lipoproteins. The affinity for T4 is greatest for TBG (approximately 1×10^{-10} M), intermediate for TTR (approximately 7×10^{-7} M) and lowest for ALB (approximately 7×10^{-5} M). Their affinity for T3 is in the same order but in each case is lower than that for T4, being approximately 5×10^{-8} M for TBG, approximately 1×10^{-7} M for TTR and approximately 1×10^{-5} M for ALB (Robbins, 1991). However the plasma concentration of each of these thyroid-hormone-binding proteins is in the opposite order, being greatest for ALB (640 μM), intermediate for TTR (4.6 μM) and lowest for TBG (0.27 μM). The combination of abundance and affinity results in the following hormone distribution in human plasma; TBG is estimated to carry 65% of bound T4 and 80% of bound T3, whilst TTR carries 15% of T4 and 9% of T3, and ALB has 20% of T4 and 11% of T3 (Robbins, 1991). It is estimated that in humans approximately one in 30000 molecules of plasma ALB has one T4 molecule bound to it, compared to one in 300 molecules of TTR and one in three molecules of TBG (Schreiber & Richardson, 1997).

The difference in the affinity of these three proteins for T4 means they can form a multicomponent 'buffer' system for T4, analogous to a multi-component pH buffer system with different pK values for each component (Schreiber & Richardson, 1997). This is illustrated in Fig. 3. Following their discovery, these proteins were called 'transport' proteins. However, the description of their role as transporters probably does not fully describe their function. Their role has been illustrated elegantly by the classic experiment of Mendel *et al.* (1987). These authors showed that when liver lobules were perfused with buffer, electrolytes and T4 (but no protein) that the T4 partitioned into the first parenchymal cells with which the perfusate came into contact, with the result that the tissue uptake of T4 was very uneven. However, when binding proteins were added to the perfusate, there was a more uniform distribution and uptake of T4 by the cells of the perfused liver. It has been suggested that the descriptor 'distributor proteins' is more apt than 'transport proteins' when applied to the plasma thyroid-hormone-binding proteins (Schreiber & Richardson, 1997). The rapid uptake of T4 is probably related to its high hydrophobicity. This hydrophobicity would likely be exacerbated by the fact that many phosholipid head groups are highly acidic. Thus, when a T4 molecule comes close to a biological membrane it enters an environment that is approximately 1.5 pH units more acid than the general medium, thus reducing the degree of ionization of the phenolic –OH group, which in turn increases the hydrophobicity of the T4 molecule and

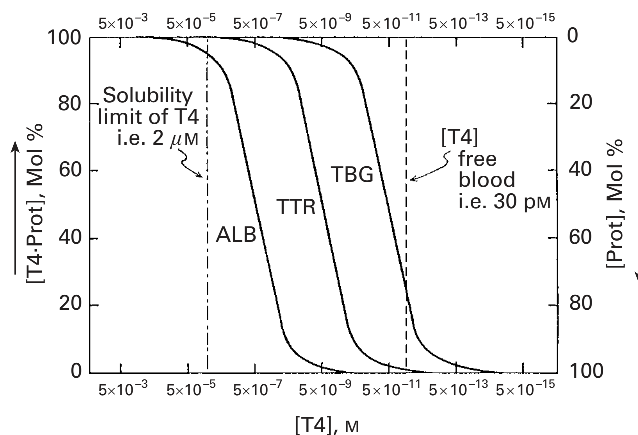

Fig. 3. The relationship between plasma free T4 concentration and T4 binding to the human plasma proteins, albumin (ALB), transthyretin (TTR) and thyroxine-binding globulin (TBG). $[T4]_{blood}$ = blood concentration of free T4, Prot = protein. Reprinted with permission from Schreiber & Richardson (1997).

hastens its partitioning into the membrane lipid bilayer (Hillier, 1970). The same argument would also apply to T3 which is more hydrophobic than T4 to begin with, having a smaller proportion of molecules with an ionized phenolic $-OH$ at physiological pH.

Although humans have three thyroid-hormone-binding proteins in their plasma this is not true for all vertebrates. An extensive survey of thyroid-hormone-binding proteins in the plasma of 93 vertebrate species (Richardson *et al.*, 1994) together with the excellent review by Schreiber & Richardson (1997) shows that ALB is the oldest thyroid-hormone-binding plasma protein, being found in all vertebrates examined. TTR is a cerebrospinal fluid protein (secreted by the choroid plexus) in adult reptiles, birds and mammals but only in adult birds, polyprotodont marsupials and eutherian mammals is it a plasma protein. This represents at least three separate occasions during the evolution of higher vertebrates where TTR became a plasma protein secreted by the adult liver. Recently, a TTR has been reported in a fish species, the sea bream *Sparus aurata* (Santos & Power, 1999).

A third T4-binding protein, migrating slower than albumin, occurs more sporadically among the vertebrates. It has been characterized in humans, rats and sheep as TBG and in the turtle as vitamin-D-binding protein. It is present in most large eutherian mammals and is probably also TBG in these species. In other vertebrate species, its precise identity is not known and it is suggested that it may be one of the apolipoproteins (Schreiber & Richardson, 1997). TBG is predominantly responsible for the high total T4 concentration in the plasma of adult humans compared to most other vertebrates (Larsson, Pettersson & Carlstrom, 1985). TBG is not present in all primate species. It is found in adults from the Catarrini and Prosimiae but not the Platyrrini (Seo *et al.*, 1989). Whilst TBG is not found in significant amounts in the plasma of adult rats or mice, it is present during the early postnatal period (Vranckx, Savu & Nunez, 1989).

In present-day adult fish, amphibians and reptiles (and presumably early vertebrates), albumin is adequate as the sole plasma thyroid-hormone-binding protein but in adult birds and most mammals there is at least one other thyroid-hormone-binding plasma protein with a higher affinity than albumin (and in the case of some eutherian mammals two such proteins). It may be that at the high metabolic activity and constant body temperatures of the endothermic vertebrates, the even distribution of thyroid hormones throughout the body required a more powerful distributor protein in their plasma.

In amphibians, TTR is a plasma protein for only a short period during metamorphosis, but not in the adult amphibian (Yamauchi *et al.*, 1998). In adult birds, diprotodont marsupials and eutherian mammals, TTR is a plasma protein. It is also secreted by the choroid plexus cells into the cerebrospinal fluid by adult reptiles, birds and mammals. Indeed, it is the predominant protein secreted by the choroid plexus in all such vertebrates examined and has been postulated to be a T4 transport system into the brain (Dickson *et al.*, 1987; Chanoine *et al.*, 1992; Southwell *et al.*, 1993). It is probably responsible for the even distribution of T4 throughout brain tissue (Schreiber & Richardson, 1997). Whilst amino acid residues at the surface of TTR show a rate of evolutionary change similar to proteins such as albumin, the central T4-binding channel of TTR is as highly conserved as the histone H4, one of the most strongly conserved proteins found in nature (Schreiber & Richardson, 1997). Although the amphibian choroid plexus does not secrete TTR into the cerebrospinal fluid bathing the brain, it does secrete a lipocalin which may have a similar function (Achen *et al.*, 1992).

In view of the findings of Mendel *et al.* (1987) and the discussion of Schreiber & Richardson (1997), it may be that in large vertebrates (such as humans) there is a requirement, because of the distances involved, for high-affinity plasma proteins to counteract the tendency for thyroid hormones to partition into membranes, and thus to ensure a uniform distribution of thyroid hormones throughout the whole body rather than a hormone gradient radiating out from the sites of T4 synthesis (the thyroid gland) and T3 manufacture (such as the thyroid, liver & kidney).

A recent observation, that in the tammar wallaby *Macropus eugenii*, there are plasma proteins which bind significant amounts of thyroid hormones during development that are not present in the adult (S. R. Richardson, unpublished data), together with the findings that TTR is secreted by the amphibian liver only during metamorphosis (Yamauchi *et al.*, 1998) and that TBG appears in the plasma for only a short period during the early postnatal life of the rat (Vranckx *et al.*, 1990), suggests it is likely that full understanding of the evolution of plasma thyroid-hormone-binding proteins in vertebrates will have to wait until more is known about their biology during vertebrate development.

The presence of multiple thyroid-hormone-binding proteins illustrates a degree of redundancy and thus a potential safety factor. For example, in documented cases where one of these plasma proteins is absent, the individuals concerned show no ill effects and appear to be healthy and exhibit normal development. This is illustrated for mice in Table 1, where although total plasma T4 concentration is reduced in TTR-null mice, they have normal free T4 and T3 plasma concentrations (Palha *et al.*, 1994). Similar deficiencies in TBG and albumin in humans, and albumin in rats, as well as other inherited or acquired variations in these proteins, still result in phenotypically euthyroid individuals (see Mendel, 1989; Bartalena & Robbins, 1992).

When studied in sheep, thyroid hormones exchanged between the plasma pool and interstitial fluids easily and appeared in the lymph faster than did plasma proteins. Free T4 levels in the plasma and lymph samples were similar (Simpson-Morgan & Sutherland, 1976) showing that free T4 concentration experienced by cells is the same as that measured as plasma free [T4].

The plasma concentration of T4 is controlled in a homeostatic manner by the secretion of TSH from the anterior pituitary. There is a diurnal cycle of TSH secretion from the anterior pituitary. In humans, peak TSH concentration occurs in the night and the nadir in the late afternoon (Fisher, 1996). Diurnal cycles have been reported for thyroid hormone levels in rats (Cokelaere *et al.*, 1996), amphibians (Gancedo *et al.*, 1997) and fish (Cerda-Reverter *et al.*, 1996; Pavlidis *et al.*, 1997).

Thyroid hormone levels are also influenced by feeding. In young pigs, levels of both T4 and T3 increased after a meal, peaking approximately 60 min after feeding; the rise was greater for T3 than T4 and was dependent on both the energy content and nutrient composition of the meal (Dauncey *et al.*, 1983). This post-meal increase involves both total and free hormone levels, is relatively immediate and although it depends on the energy content of the food, is not directly related to changes in blood glucose levels. It is also observed in thyroidectomized animals and likely related to changes in peripheral deiodination and redistribution of hormonal pools (Dauncey & Morovat, 1993). The circadian rhythms in plasma thyroid hormone levels in rats are influenced by their feeding pattern and are most pronounced when the rats are fed only once a day (Cokelaere *et al.*, 1996). The diurnal cycle in thyroid hormone concentrations is influenced by nutritional state in fish (Cerda-Reverter *et al.*, 1996) and food deprivation results in reduced serum levels of thyroid hormones in horses (Messer *et al.*, 1995) and of T4 in penguins (Groscolas & Leloup, 1989).

As well as food deprivation, non-thyroidal illness can also reduce plasma levels of thyroid hormones, especially T3. In humans, T4 concentrations change very little, whilst those of T3 decrease and rT3 increase during starvation (Danforth, 1986). Similar changes occur during non-thyroidal illness (Kaptein, 1986). In cats, non-thyroidal illness results in a significantly lower total T4 concentration but no change in free T4 levels (Mooney, Little & Macrae, 1996).

Blood levels of T4 represent the balance between the rate of hormone secretion and disposal. Changes in plasma levels may reflect changes in the regulatory system or may simply reflect changes in the steady state. Changes in total levels need not reflect changes in thyroid status. For example, during pregnancy in humans, total T4 concentration increases due to elevated serum TBG levels, however free T4 concentration remains in the normal non-pregnant range (O'Leary *et al.*, 1992). Most reported changes are for total hormone levels and whether there are similar changes in free levels is often not known. Indeed, with such a large protein-bound fraction in the plasma, substantial changes in free T4 levels are probably not easily achieved over short periods of time.

Since T4 disposal appears related to metabolic rate (see Section II.3), some reported changes in thyroid hormone levels may best be interpreted as changes in the steady state following changes in metabolic rate. For example, in the rat, cold exposure results in increased T4 degradation and a decrease in plasma total T4 levels (Gregerman, 1963). That thyroid secretion rate also increases during cold exposure (Lachiver & Petrovic, 1960) is probably best explained as a homeostatic response to maintain relatively constant plasma T4 levels in the cold, rather than a hormonal response to increase heat production in the cold. If the latter were the case we would expect to measure an increase in plasma T4 levels rather than a decrease.

Chronic exercise (swimming) in rats results in no change in either total or free levels of T4 or T3 (Woody *et al.*, 1998). Exercise in thoroughbred horses resulted in no change in total T4 levels but an increase in total T3 concentration (Gonzalez *et al.*, 1998). A 48 km flight in pigeons resulted in a 35% decrease in total T4, a 60% decrease in total T3 and a 130% increase in rT3 levels (George & John, 1992).

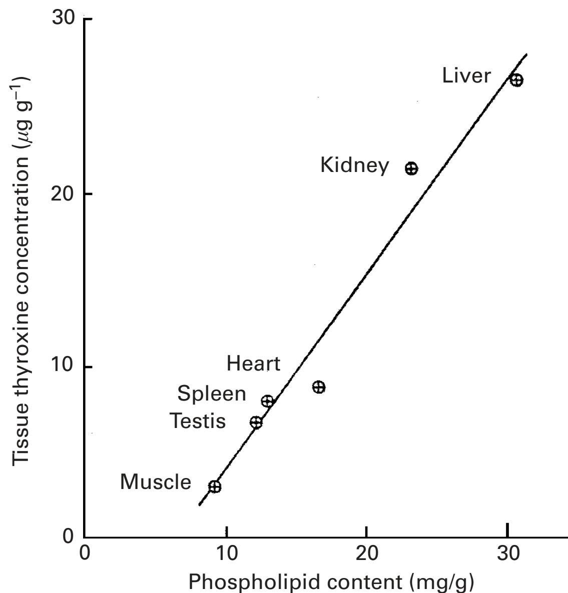

Fig. 4. The relationship between phospholipid content and thyroxine (T4) content of various tissues in the rat. Reprinted with permission from Hillier (1970).

An examination of seasonal variations in hormone concentrations of humans showed a small but significant variation in total T3 but none in free T4 levels (Maes *et al.*, 1997). Prolonged residence in Antarctica resulted in no significant change in either total or free T4 levels in humans but did result in a significant decrease in both total and free plasma concentration of T3 (Reed *et al.*, 1986).

Animals that do show substantial seasonal variation are often those that have a seasonal inactive (hibernation) phase. For example, woodchucks (*Marmota monax*) show a seasonal variation in free T4 levels with low values at the autumnal equinox (Rawson *et al.*, 1998) and hedgehogs *Erinaceus europaeus* show a low level of total T4 during winter (Fowler, 1988). In the black bear *Ursus americanus*, which has an inactive period known as hibernation (without the dramatically depressed body temperatures of other mammalian hibernators) there is a small decrease in free T4 and free T3 levels which seems to be related to food restriction (Tomasi, Hellgren & Tucker, 1998). In reptiles, both the viper *Vipera aspis* (Naulleau, Fleury & Boissin, 1987) and the desert iguanid *Dipsosaurus dorsalis* (John-Alder, 1984*b*) have a low total T4 concentration during their annual hibernation period, whereas in the non-hibernating green sea turtle *Chelonia mydas*, plasma total T4 concentration is constant throughout the year (Licht, Wood & Wood, 1985). In a study of three diverse turtle species, two species (green turtle *Chelonia mydas* & Mexican tortoise *Gopherus flavomarginatus*) had low plasma T4 levels with no significant seasonality, whilst the painted turtle *Chrysemys picta* showed significant seasonal variation in plasma total T4 levels with the lowest values associated with the hibernation period (Licht *et al*,. 1991).

In general, thyroid hormone plasma concentrations appear much less labile than the levels for many other hormones. Often the changes measured in total hormone concentration reflect changes in thyroid-hormone-binding proteins rather than changes in thyroid status. The protein-bound thyroid hormones in the plasma represent a significant hormonal store that will act to dampen changes in the hormone content of some tissues. For example, when rats are thyroidectomized, within two weeks plasma concentrations of T4 and T3 fall dramatically by 96-98 % whilst muscle, heart and brain T4 and T3 contents only fall by approximately 50 % over the same period (Obregon *et al.*, 1981). Thyroid hormone concentrations appear most variable between fish species.

(3) Cellular uptake, cellular location and hormone metabolism

In 1968, Hillier examined the uptake and release of T4 and T3 by the isolated perfused rat heart. Initial rates of uptake were similar for both T4 and T3, whilst a steady state was reached after 30 min perfusion for T4 and after 120 min for T3. T3 equilibrated to a 'space' five times that of T4 (which was 15 ml g^{-1} heart). This 'space' was independent of perfusate hormone concentration (13 pM to 1.3 μM), and the rates of equilibration were relatively unaffected by temperature. Washout curves suggested two compartments, a fast-release compartment which was the same size for T4 and T3 and a slow-release compartment which was much greater for T3 than T4 (Hillier, 1968*b*). These results suggested a purely physical process of partitioning between the perfusate and a compartment within the heart in which the hormones are much more soluble (T3 more so than T4). He also showed, that with serum (containing hormone-binding proteins) in the perfusate, the uptake of hormones was reduced (more so with T4 than T3), that hormone uptake became more dependent on perfusate concentration and that the release of accumulated hormone was also accelerated (Hillier, 1968*a*). Two years later, in a follow-up study examining the binding of thyroid hormones to phospholipid membranes he showed

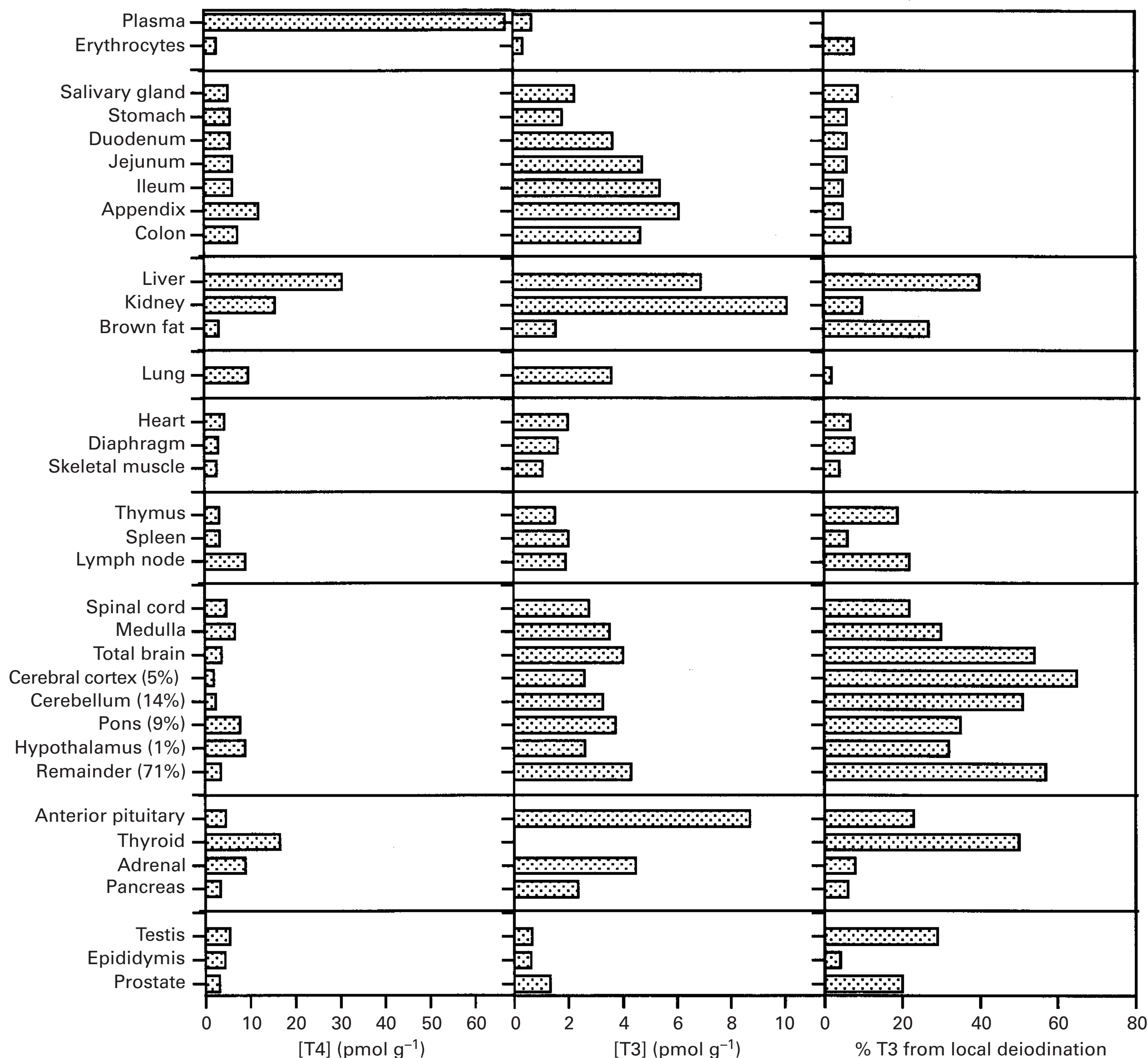

Fig. 5. T4 and T3 content of tissues from the euthyroid rat. The % T3 formed from local deiodination of T4 is also shown. All data are taken from van Doorn *et al.* (1985).

that there was a strong correlation between the phospholipid content of different tissues and the tissue T4 concentration of the same tissues. This relationship is shown in Fig. 4.

Since the studies of Hillier (1968*a*, *b*, 1970) the tissue concentrations of T4 and T3 have been measured both by isotopic equilibrium analysis and radioimmunoassay of extracted hormones with the values obtained being similar for the two techniques (Obregon, Morreale de Escobar & Escobar del Rey, 1978). In Fig. 5 the T4 and T3 tissue concentrations for the rat are shown, including the % T3 formed from local deiodination of T4 (van Doorn, Roelfsema & van der Heide, 1985). The T4 and T3 contents of most tissues are in the 1–10 pmol g^{-1} range, with the liver and kidney having higher tissue T4 contents of 31 pmol g^{-1} and 16 pmol g^{-1}, respectively. Liver and kidney T3 contents were more typical of other tissues being, respectively, 7 pmol g^{-1} and 10 pmol g^{-1}. It is of interest that the liver has more T4 per gram than does the thyroid gland where most thyroid hormones are found in the form of thyroglobulin. The plasma has the highest T4 content but most of this is protein-bound with the free concentration being only

approximately 20 pM (equivalent to approximately 0.02 pmol g^{-1}). It is not possible directly to translate tissue contents in pmol g^{-1} to equivalent concentrations because cells are not homogenous but contain compartments such as membranes, in which iodothyronines have a much greater solubility, as well as cellular proteins to which they can bind.

Traditionally, intestinal contents have been excluded in studies analyzing the tissue distribution of thyroid hormones (including Fig. 5) but recent studies show that there are significant quantities of both T4 and T3 in intestinal contents and that these are readily exchangeable with other body compartments (Nguyen *et al.*, 1993).

The thyroid hormones with non-ionized phenolic groups are amphipathic molecules; although they are highly soluble in phospholipids, spin-label studies suggest that T3 does not flip-flop across membranes to any appreciable extent and will tend to remain in the outer half of the bilayer (Lai *et al.*, 1985; although an effect of the spin label itself influencing the property measured cannot be ruled out in this particular study). Interestingly, the spin-labelled T3 was measured to have a lateral diffusion constant in the membrane of 3×10^{-8} cm^2 s^{-1} at 31 °C (Lai & Cheng, 1984) which is similar to the estimated rate of diffusion through cytoplasm for T3 (Luxon & Weisinger, 1992). The thyroid hormones are likely to enter cells by a number of possible means, including in some situations by endocytosis of protein-bound thyroid hormones, as well as by specific carriers. They can be taken up into many cells by an energy-dependent transport system located in the plasma membrane (Rao *et al.*, 1976; Krenning *et al.*, 1978). The membrane iodothyronine transporters have been reviewed by Kragie (1994, 1996).

Many uptake studies do not take into account the partitioning of thyroid hormones into lipid membranes in their measurement of uptake. Studies that show saturation suggests a carrier-mediated process and the use of various inhibitors suggest that uptake is often energy-dependent and sometimes also dependent on the transmembrane Na^+ gradient. Uptake is also often shown to be stereospecific. In the rat liver cell, there appears to be at least two carriers, one which preferentially transports T4 and the other transporting T3 (Krenning & Docter, 1986). Although less than half the T3 found in liver comes directly from local deiodination (Larsen, Silva & Kaplan, 1981; see Fig. 5), the tissue is an important source of systemic T3 (from the deiodination of T4). There is thus also a substantial efflux of T3 (and T4) from the liver to the plasma which appears to be passive (Krenning & Docter, 1986). Carrier-mediated, saturable uptake of thyroid hormones has also been described for isolated adipocytes (Parl *et al.*, 1977; Landeta, Gonzalez-Padrones & Rodriguez-Fernandez, 1987), cultured fibroblasts (Cheng *et al.*, 1980), cultured pituitary tumour (GH3) cells (Horiuchi *et al.*, 1982), lymphocytes (Holm *et al.*, 1980) and more recently in cultured cells from rat anterior pituitary where T3 and T4 may enter by the same carrier (Everts *et al.*, 1993, 1994). It has recently been demonstrated in neonatal rat cardiac myocytes (Everts *et al.*, 1996) but not in cultured human muscle cells (Bolhuis *et al.*, 1983), in cultured glial C6 cells (Yusta *et al.*, 1988) nor in choroid plexus (Dickson *et al.*, 1987). It seems that thyroid hormone uptake is controlled differently in hepatocytes and anterior pituitary cells (Everts *et al.*, 1995). Many studies suggest an interaction between amino acid transporters and the iodothyronines and some suggest the iodothyronine transporter is either a previously described amino acid or neurotransmitter transporter. It may be that iodothyronine transporters and these other transporters are related. In some situations, it appears that plasma membrane transport is an important limiting factor for further metabolism of thyroid hormones within cells (for review see Hennemann *et al.*, 1998)

Thyroxine uptake by hepatocytes from juvenile rainbow trout *Oncorhynchus mykiss* suggests that the basic system in fish liver cells closely resembles that observed for mammalian hepatocytes, in that it is a stereospecific, saturable, energy-dependent carrier-mediated process. In these fish hepatocytes, thyroxine uptake does not depend on extracellullar Na^+ but may involve an endocytotic component (Riley and Eales, 1993).

Of all body tissues of the rat, the brain shows the greatest contribution of tissue T3 derived from local deiodination (see Fig. 5). It has a specialized system of T4 uptake *via* the unidirectional secretion of newly synthesised TTR by the choroid plexus, that enables T4 to cross the blood-brain barrier (Dickson *et al.*, 1987). Labelled T4 is rapidly accumulated by the choroid plexus both *in vivo* and *in vitro*. This process appears to be simple partitioning between the blood plasma and the choroid plexus membranes. Thus, the choroid plexus acts as a transport system for T4 to pass from the blood to the brain *via* the cerebrospinal fluid (Dickson *et al.*, 1987).

Once taken up into cells, thyroid hormones can be found either (i) 'free' in aqueous solution, (ii) 'bound' to cellular proteins or (iii) located within

the lipid matrix of the various cellular membranes. They will be in some form of steady state between all three classes of sites. I know of no work that has attempted, in any cell type, to determine the relative amounts of either thyroid hormone in each of these three locations.

As determined by differential centrifugation of tissue homogenate, the subcellular distribution of ^{125}I-labelled T4 in normal rat liver is 54, 18, 17 and 11% in microsomes, supernatant, nuclei and mitochondria respectively, whilst the corresponding protein distribution is 25, 37, 23 and 15% respectively (Schwartz, Bernstein & Oppenheimer, 1969). The subcellular location of thyroid hormones has also been assessed in some tissues by the use of autoradiography. In cultures of developing nervous tissue, Manuelidis (1972) showed that ^{125}I-labelled T4 accumulated at the cell membrane, mitochondria, endoplasmic reticulum, nucleus and the synapse in order of decreasing intensity. Following the injection of 0.3–1.0 nmol of ^{125}I-labelled T3 into the rat, Bar-Sella, Stein & Gross (1973) examined its sub-cellular localization in the posterior pituitary and median eminence. The 'subcellular sites of hormone concentration were found to correspond to intracellular membrane structures, such as the mitochondria, Golgi apparatus and nuclear envelope' and 'many of the grains counted with the nucleus are actually associated with the nuclear envelope and some of the grains counted with the cytoplasm may be due to labelled hormone localized on the plasma membrane' (Bar-Sella *et al.*, 1973, p. 1412). In isolated liver cells, autoradiography of labelled T3 showed that the hormone was subcellularly associated with mitochondria, endoplasmic reticulum, cytoplasm, nuclei, lysosomes and lipid in decreasing intensity (Sterling *et al.*, 1984). Because of the physics and geometry involved in transmission electron microscopy, it is only possible to view membranes along the plane of the bilayer and not perpendicular to the plane of the bilayer. Thus, it is possible that some of the T3 molecules assigned to the cytoplasm may actually be membrane located (e.g. in endoplasmic reticulum that is in the plane of the section). Thus, electron microscopic autoradiography will underestimate the association of thyroid hormones (and other amphipathic molecules) with membranes because we are not able to 'see' all cellular and subcellular membranes. Their relative absence in lipid droplets in cells may reflect the fact that the hormones are amphipathic rather than completely hydrophobic and thus they are more likely to be associated with the interface between lipid droplets and surrounding aqueous environment rather than evenly distributed in the lipid body. Kriz, Fong & Goldfine (1981) also reported evidence for the localization of T3 at multiple cellular sites.

Hillier (1970) found that in rat liver homogenate 12–15% of T4 was present in the 'free' state (in the aqueous compartment). Simple alcoholic extracts indicated that at least half of the T4-binding capacity of the homogenates was due to lipid material and boiling the homogenate (which will denature binding proteins but not significantly affect the relatively thermostable phospholipids) did not impair its ability to bind T4. The influence of this hydrophobic compartment of the cell (i.e. its membranes) on the distribution of thyroid hormones is shown by the correlation between tissue phospholipid content and tissue T4 content (see Fig. 4).

Measurements of T4 and T3 concentration in human erythrocytes show that 60% of T3 and 34% of T4 were associated with the cell membrane even after seven washes of erythrocyte 'ghosts' (Bregengaard *et al*, 1989). The membrane can be calculated to account for approximately 1% of the total cell volume using values for the volume (94 fl) and membrane surface area (135 μm^2) of an average human erythrocyte (Evans & Fung, 1972). These amphipathic hormone molecules are largely associated with the membrane bilayer because of their physical chemistry (and thus not 'bound' to it in the normal sense). These measurements are likely to be underestimates in light of the extensive washing of the membrane preparations prior to the measurement process. The membrane component will include T4 and T3 both in the lipid matrix of the bilayer as well as that bound to membrane proteins. The fact that the membrane to cytosol ratio for the various iodothyronines was the same in euthyroidism, hyperthyroidism and hypothyroidism suggests a purely physical relationship with thyroid hormones possibly concentrated mostly in the lipid bilayer. High-affinity binding sites have also been measured in both human and rat erythrocyte membranes (Angel, Botta & Farias, 1989)

Although the amount of thyroid hormones in membranes may not be great, because of their relatively small volume compared to the aqueous compartment of cells, the concentration of thyroid hormones in membranes will be many times greater than that in the aqueous compartment of the cell.

Both T4 and T3 bind to a number of cellular proteins. In the euthyroid rat, it is estimated that less than 15% of tissue T3 is bound to the specific

nuclear receptors, except in the anterior pituitary where it is approximately 50% (Oppenheimer, Schwartz & Surks, 1974). How much of the extranuclear T4 and T3 is bound to extranuclear proteins is unknown. Extranuclear binding sites are located on plasma membranes, in the mitochondria and in the cytosol and many have a high affinity for T3. For a list of extranuclear binding sites and their respective affinities see Kragie (1996) and for plasma membrane binding sites see Segal (1989*b*). Binding to the nuclear envelope has also been reported (Lefebvre & Venkatraman, 1984) and a protein located on the luminal face of the endoplasmic reticulum and nuclear envelope also binds thyroid hormones (Kato, Velu & Cheng, 1989*b*). One cytoplasmic binding protein is the monomer of pyruvate kinase subtype M_2, that can bind either T4 or T3; binding, in turn, inhibits the enzymatic activity of the monomer. This enzyme also exists in a tetrameric form which has greater enzymatic activity than the monomeric form but the tetrameric form does not bind thyroid hormones (Kato *et al.*, 1989*a*).

Very little is known about either the specific identity of most of these proteins or the physiological importance of the binding. Some of them may be involved in the transport and the metabolism of the thyroid hormones whilst others may have their functions affected by such binding and thus mediate some thyroid hormone effects. In rat liver, one of the cytosolic binding sites has been identified as the glutathione-S-tranferase enzymes and although the thyroid hormones are potent inhibitors of the activities of these enzymes this may not be physiologically important in the euthyroid state (Ishigaki, Abramovitz & Listowsky, 1989). The glutathione-S-transferase enzymes are known to bind many different lipophilic compounds (Mannervik, 1985). That much of thyroid hormone binding to proteins involves hydrophobic interactions is illustrated by the fact that fatty acids are capable of competitively inhibiting binding both to plasma proteins (e.g. Lim *et al.*, 1988) and to the nucleus (Wiersinga, Chopra & Teco, 1988; Inoue *et al.*, 1989; van der Klis, Wiersinga & de Vijlder, 1989).

Amphipathic molecules diffuse through hepatic cytoplasm relatively slowly. Estimation of the intracellular transport rate of T3 suggests a rate of approximately 3×10^{-8} cm^2 s^{-1} (Luxon & Weisinger, 1992). This is approximately the same rate as that measured for the lateral diffusion of T3 in membrane bilayers (Lai & Cheng, 1982, 1984) and is also typical of aqueous diffusion of either amphipathic or protein-bound molecules. The measured rate for a saturated fatty acid analogue is approximately 0.4×10^{-8} cm^2 s^{-1} (Weisinger, 1996).

Intracellular T4 and T3 undergo various metabolic transformations. Knowledge of these metabolic transformations and their relative importance in different tissues has exploded over the last two decades and is reviewed in detail elsewhere (see Hennemann, 1986; Kohrle, Brabant & Hesch, 1987; Berry & Larsen, 1992; Leonard & Koehrle, 1996; St. Germain & Galton, 1997). Most is known about the various deiodinative pathways which are responsible for approximately 80% of the daily T4 breakdown in humans with non-deiodinative pathways (conjugation, deamination and decarboxylation as well as oxidative degradation) responsible for the remaining 20% (Burger, 1986). The non-deiodinative pathways may be responsible for up to approximately 56% of T3 metabolism in humans (Burger, 1986). The pathways of thyroid hormone deiodination are shown in Fig. 6.

Monodeiodination of T4 results in the removal of iodine from either the outer (phenolic) ring or inner (tyrosyl) ring and produces T3 or rT3, respectively. These two molecules can be further deiodinated to three types of diiodothyronines (3,5-T2, 3,3′-T2 or 3′,5′-T2), which in turn can be deiodinated to form two monoiodothyronines (3-T1 or 3′-T1), which can be converted to the non-iodinated thyronine (see Fig. 6). There are three separate types of deiodinases, initially differentiated by kinetics and patterns of inhibition etc., they can now be identified by their cDNAs. They have been called type I, type II and type III, and more recently D1, D2 and D3 (St Germain & Galton, 1997). All three appear to have a selenocysteine residue at the active site of the enzyme, with selenium being very important in determining catalytic efficiency (Berry & Larsen, 1992). All require cytosolic thiols for their activity and they possess both different affinities for substrates and have different relative affinities for the range of iodothyronines. All three deiodinase enzymes are membrane-bound enzymes located mainly in the microsomal fraction of tissue homogenates suggesting an endoplasmic reticulum and/or plasma membrane location.

D1 is probably found in all tissues but has especially high activity in liver, kidney, thyroid tissue and the central nervous system. In kidney, D1 is found on the basolateral plasma membrane of proximal convoluted tubule cells, whilst in the liver it is located on the endoplasmic reticulum. It is capable of both outer- and inner-ring deiodination

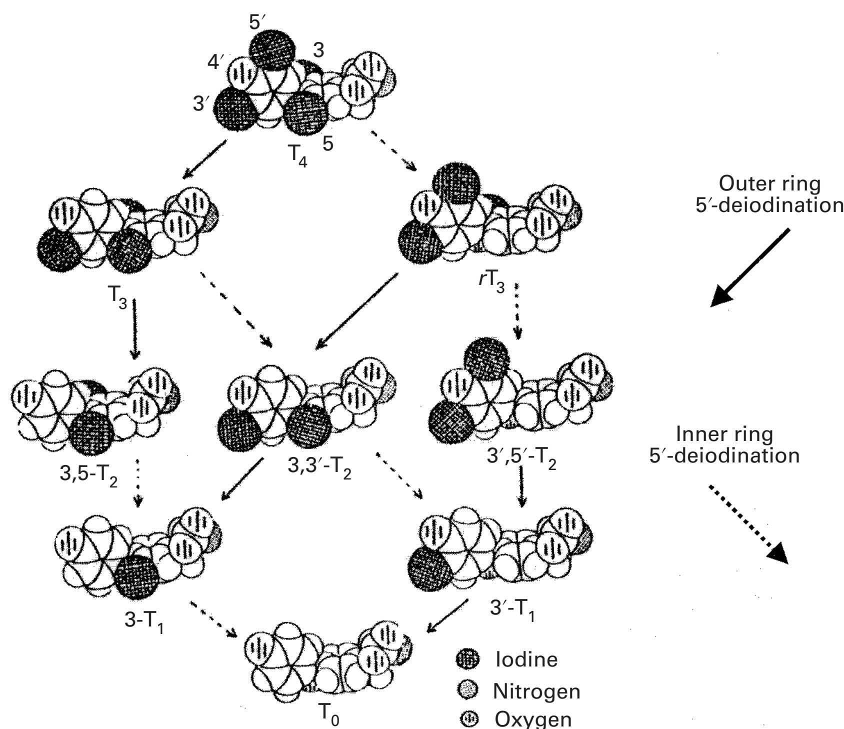

Fig. 6. Pathways of the deiodination cascade of T4 resulting in the various iodothyronines. Solid arrows represent outer ring monodeiodination and broken arrows represent inner ring monodeiodination. Reprinted with permission from Kohrle *et al.* (1986).

and in many animals is inhibited by the thyroid inhibitor propylthiouracil (PTU). A PTU-insensitive D1 deiodinase has recently been described for a teleost fish species (Sanders *et al.*, 1997).

D2 has been found in the central nervous system, brown adipose tissue, anterior pituitary and placenta and is capable of outer-ring deiodination. It has a higher affinity for T4 than does D1, and is not inhibited by PTU. D2 is located on the plasma membrane in the cerebral cortex. *In situ* hybridization histochemistry in the rat hypothalamus and pituitary shows that D2 mRNA is not evenly distributed but is heavily concentrated in tanycytes, which are glial cells that reside in the floor and lateral walls of the third ventricle having long cytoplasmic processes that extend into the adjacent neuropil of the medial hypothalamus and median eminence where they are intimately associated with axon terminals and blood vessels (Tu *et al.*, 1997). In the neonatal rat brain, the D2 deiodinase is primarily expressed in glial cells, rather than neurones (Guadano-Ferraz *et al.*, 1997). In the adult rat brain, the choroid plexus lacks deiodinase activity (Southwell *et al.*, 1993).

D3 is found in the central nervous system and the placenta and carries out inner-ring deiodination. It is also not inhibited by PTU. The D3 isolated from human placenta has been shown to have an essential requirement of phospholipids for activity (Santini *et al.*, 1992).

All three deiodinases are evolutionarily quite old: cDNAs have been isolated for all three types from various mammalian, avian, amphibian and fish species and an evolutionary tree determined (Fig. 7). The separation of the three deiodinase enzymes predates the separation of the ancestors of extant vertebrates. The deiodinases are related to each other and their relative tissue distribution varies between vertebrate species. It has become obvious that these deiodinases are an integral part of the vertebrate thyroid hormone axis. They are themselves influenced by thyroid status and also appear to be important during development. The tissue distribution of the various deiodinases suggests that they are responsible for the intracellular concentrations of thyroid hormones in different tissues. Their importance in determining tissue-specific intracellular concentrations of T4 and T3 is illustrated by the recent finding that in hypothyroid rats, euthyroidism in all tissues can only be achieved by the combined infusion of both T4 and T3 at approximately the relative rates that both T4 and T3 are secreted by the rat thyroid gland: 1.2 nmol T4 and 0.23 nmol T3/100 g/day (Escobar-Morreale

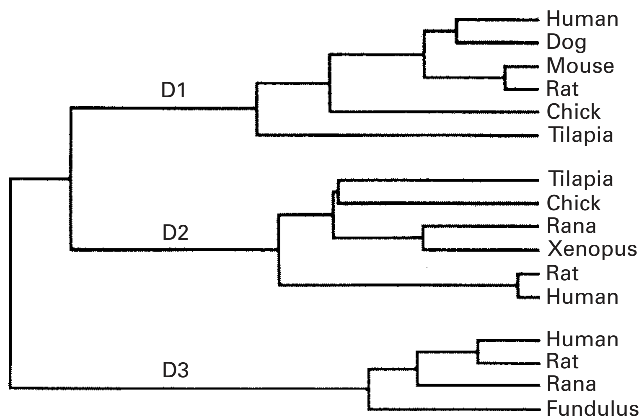

Fig. 7. Evolutionary tree diagram of the deiodinase enzymes (D1, D2, D3) from various vertebrates. Reprinted with permission from St. Germain & Galton (1997).

et al., 1996). The deiodinases are also involved in the autoregulation of T3 at the cellular level during periods of altered plasma concentrations of thyroid hormones, especially in the brain (Dratman *et al.*, 1983; St. Germain & Galton, 1997).

The presence of intracellular deiodinases results in the fact that most of the body's T3 is produced by deiodination of T4. It is estimated that, in the rat, approximately 55% of the circulating T3 comes from the thyroid gland, with a significant amount of this being produced by the intrathyroidal deiodination of T4 by the thyroid's own D1 deiodinase (Chanoine *et al.*, 1993*a*). The remainder of the circulating T3 comes predominantly from the liver and kidney. Whereas dietary selenium deficiency in the rat results in a dramatic decline in both liver selenium content and liver D1 deiodinase activity, the decline in thyroid selenium content is modest and thyroid D1 deiodinase actually increases in activity (Chanoine *et al.*, 1993*a*).

The relative importance of T3 derived from local cellular deiodination for the T3 content of tissues varies from 2–4% in lung and skeletal muscle to 65% in cerebral cortex (see Fig. 5).

Deiodination is also responsible for the other iodothyronines found in vertebrates. In human serum, total rT3 is approximately 600 pM, the three T2s are 55–85 pM, and T1 is approximately 60 pM (Chopra, 1996). The total serum concentrations of Tetrac and Triac (the deaminated products of T4 and T3) are 7.2 nM and 2.8 nM respectively whilst the sulphated conjugates of T4 and T3 are, respectively, approximately 20 pM and approximately 75 pM (Chopra, 1996). The free concentrations of iodothyronines in adult human serum (measured by ultrafiltration) are 30 pM T4, 4.8 pM T3, 0.6 pM rT3, 0.4 pM 3,3′-T2 and 0.8 pM 3′,5′-T2 (Faber *et al.*, 1984). We know very little of the concentration of these iodothyronines in other vertebrates.

The iodothyronines produced from T4 and T3 are less lipophilic than either T4 or T3. Information regarding their relative hydrophobicity can be garnered from their partitioning between the erythrocyte cytoplasm and membrane. In human erythrocyte ghosts, the following percentages of iodothyronines partition into the membrane; 60 % for T3, 34% for T4, 23% for 3,3′-T2, 16% for rT3 and 3% for 3′,5′-T2, with these values being the same in hypo- and hyperthyroidism (Bregengaard *et al.*, 1989). The order of these iodothyronines is similar to their expected hydrophobicity at physiological pH, when both the lipophilicity of the iodine (the more iodine atoms the more hydrophobic the molecule) together with the degree of ionization of the phenolic OH group (the more iodines on the phenolic ring the less hydrophobic the molecule) are taken into account. The calculated phospholipid:cytoplasm partition ratios (assuming the cell membrane is 1% of total cell volume) for T3, T4, 3,3′-T2, rT3 and 3′,5′-T2 are approximately 150, 50, 30, 20 and 3, respectively. These values are likely underestimates because of the seven washes of the 'ghosts' carried out before measurement (Bregengaard *et al.*, 1989).

In normal euthyroid humans (70 kg body mass), the production rates of the iodothyronines are estimated to be 130 nmol day^{-1} for T4, 48 nmol day^{-1} for T3, 60 nmol day^{-1} for rT3, 50 nmol day^{-1} for 3,3′-T2, 21 nmol day^{-1} for 3′,5′-T2, 7 nmol day^{-1} for 3,5-T2 and 30 nmol day^{-1} for 3′-T1 (Chopra, 1996). The deamination products of T4 and T3 are produced at only 1.8 and 5.8 nmol day^{-1} respectively, whilst 10 nmol day^{-1} is the production rate of the T3 sulphate conjugate (Chopra, 1996).

In a study of the turnover of six iodothyronines (T4, T3, rT3, 3,3′-T2, 3′5′-T2 and 3′T1) in the euthyroid rat, DiStefano & Feng (1988) found the distribution of all six iodothyronines could best be characterized by three pools (in increasing size); a plasma pool, a rapidly exchanging pool and a slowly exchanging pool. The respective pool sizes for T4 (as % of total pool size) were approximately 25, 18, and 57, whilst for T3 they were 2, 24 and 74. They were similar for the other four iodothyronines, being approximately 3–9, 8–22 and 60–90% of total pool size for the plasma, rapidly exchanging and slowly exchanging pools, respectively. The precise identity of these two non-plasma pools is not known but there appear to be at least two possibilities; they could

reflect the tissue distribution of the thyroid hormone uptake mechanisms into two groups or they could represent two physical compartments, respectively, an aqueous non-membrane compartment and a lipophilic membrane compartment. The fact that the slowly exchanging pools are the major sites of T4 monodeiodination suggests the second scenario.

In the rat, faecal excretion accounts for 24 % of T4, 30 % of T3, and 6 % of T1 turnover but only 1–3 % of disposal of rT3 and the T2s. The remaining portion of each iodothyronine was completely and irreversibly metabolised. The kinetic variables for all the iodothyronines except T4 and T3 were similar, suggesting that similar mechanisms are responsible for their transport, metabolism and distribution. The production rates (per 100 g body mass) are approximately 1 nmol T4 day^{-1}, approximately 0.25 nmol T3 day^{-1} and 0.13–0.84 nmol rT3 day^{-1} (DiStefano & Feng, 1988).

Expressed per 100 g body mass, the human production rate is 0.19 nmol T4 day^{-1} and 0.07 nmol T3 day^{-1}. Both these rates are 20–25 % of the respective production rates measured for the rat and illustrate the body size *versus* metabolism relationship in mammals (see Kleiber, 1961). The basal metabolic rate (BMR) of mammals is proportional to body mass to the 0.73 power. A recent compilation of T4 and T3 utilization rates by euthyroid mammals (which for T4 equals the hormonal production rate) has shown that these rates scale to body mass to the 0.74 and 0.81 power, respectively (Tomasi, 1991). Thus in mammals there is a correlation between the BMR and the turnover of T4 (and T4 secretion from the thyroid). But what is cause and what is effect? Is the difference in BMR due to the difference in T4 secretion rate or is the T4 secretion rate due to the difference in BMR? Because the hypothalamic/anterior pituitary/thyroid axis is organized to maintain a constant plasma T4 concentration it is possible that the thyroidal T4 secretion rate varies with the 0.74 power of body mass because the metabolic disposal of T4 is related to the overall metabolic rate of the mammal (which is proportional to the 0.73 power of body mass). Insight can be gained from Fig. 2. If thyroidal T4 secretion is the 'cause' of the body-mass-related change in BMR then we might expect that plasma concentrations of free thyroid hormones would be greater in smaller mammals because of their greater mass-specific metabolic rate, since presumably the regulatory information 'seen' by the cells is the free hormone concentration. There is no such allometric trend in free hormone levels in mammals. Thus, a reasonable conclusion is that the variation in BMR is more likely the 'cause' of the observed variation in T4 secretion rates in mammals rather than *vice versa*. This does not preclude the fact that thyroid status (which is related to the concentration of the thyroid hormones rather than their turnover) influences the metabolic rate of mammals specifically, and vertebrates in general.

It was originally thought that the metabolism (especially deiodination) of thyroid hormones was involved in the exertion of thyroid hormone effects; however, because of the dominance of the nuclear receptor paradigm as the only accepted mode of thyroid hormone action, this idea has not received much attention. Early work (Oppenheimer *et al.*, 1971) that is cited as evidence for a dissociation between hormonal deiodination and hormonal action is not valid if these hormones act at more than one site in the cell. The phenobarbital-induced increase in T4 deiodination used by the above study is due to an increased amount of microsomal membrane per cell and would likely result in a decreased T4 availability for other sites in the cell. Thus, if T4 acts at multiple sites in the cell, as I will argue later, we would expect a significant decreased effect of T4 at these other sites also. This is exactly what was observed by Oppenheimer *et al.* (1971); the two effects measured were resting oxygen consumption and the activity of the mitochondrial enzyme, glycerolphosphate dehydrogenase. If there are multiple sites of action in the cell, the possibility that deiodination is involved in some thyroid hormone effects should remain open.

In humans, low-energy diets lead to changes in both deiodinative and non-deiodinative pathways with consequent changes in plasma iodothyronine levels. Serum levels of free T4, total 3,3′-T2 and 3′,5′-T2 are unaffected, whilst serum free T3 and total rT3 concentrations are reduced and serum total 3,5-T2 concentrations are increased with a low-energy diet. There is a decrease in the overall plasma disposal rate, but no change in the percentage of T3 metabolized by inner and outer ring monodeiodination. For rT3 there is a large decrease in outer ring deiodination, a small increase in inner ring deiodination and a large increase in non-deiodinative metabolism with a low-energy diet (Burger *et al.*, 1987). That dietary-induced alterations in deiodinases can lead to complex alterations in the thyroid axis is illustrated by the finding that fasting leads to an increase in D2 deiodinase activity in the rat hypothalamus (Diano *et al.*, 1998). In young pigs, low energy intake results in lowered serum T4 and T3 concentrations but low ambient temperature has

no effect on serum hormone levels. However, whilst low temperature results in a decreased fractional turnover of T4 and T3, energy intake has no effect (Macari *et al*., 1983).

Variations in the metabolism of thyroid hormones have also been observed during non-thyroidal illness in humans with the degree of thyroid hormone disturbance being correlated with disease severity. Commonly during such illnesses, serum T3 levels are lowered and sometimes T4 levels and TSH levels are reduced as well (McIver & Gorman, 1997) and serum 3,5-T2 is elevated (Pinna *et al*., 1997). In some illnesses, there is also interference with plasma-protein binding as well as cellular uptake of thyroid hormones (Lim, Stockigt & Hennemann, 1995).

III. EFFECTS OF THYROID HORMONES

(1) When studying the effects of thyroid hormones

The first question is which thyroid hormone to use. It is a common assumption in the literature that T3 is the 'active' thyroid hormone and that T4 is only a prohormone. It is not uncommon to see T4 referred to as the 'inactive' hormone. Whilst T3 is more active on a 'per mole' basis in many situations, this does not mean T4 is 'inactive'. Indeed, T4 is the predominant secretion by the thyroid gland and a case can be made that T4 is the more physiologically relevant hormone for *in vivo* studies, as it allows the body's cells to perform any appropriate deiodinations. T4 is an active hormone in its own right. T4 has been reported to affect directly the expression of thyroid-hormone-sensitive genes (Bogazzi *et al*., 1997).

T4 also can have effects separate from T3. For some effects, T4 has been shown to be two orders of magnitude more potent than T3 (Leonard, Siegrist-Kaiser & Zuckerman, 1990; Farwell, Tranter & Leonard, 1995). Similarly, studies on birds show that T3 cannot substitute for T4 regarding reproductive timing (Pant & Chandola-Saklani, 1995; Reinert & Wilson, 1997). The use of T3 rather than T4 may be misguided and based upon an assumption (T4 is only a prohormone) that is not valid in many situations.

The importance of taking into account both cellular uptake mechanisms and cellular deiodinase systems is illustrated by the recent finding that, in the thyroidectomized rat, normal euthyroid intracellular contents of T4 and T3 were only achieved when both T4 and T3 were infused at the rates they are reported to be secreted from the thyroid gland (Escobar-Morreale *et al*., 1996).

In a similar vein, other iodothyronines have also been shown to have significant effects. For example, rT3 often regarded as solely an 'inactivated' thyroid hormone, is sometimes more potent than T3 (e.g. Leonard *et al*., 1990; Farwell *et al*., 1995). It has also been shown that 3,5-T2 is an 'active' thyroid hormone with respect to the calorigenic action of the thyroid hormones (Horst, Rokos & Seitz, 1989).

However, because of the common practice of using only T3 when studying thyroid hormone effects both *in vitro* and *in vivo* much of the remainder of this review will consider the effects of thyroid hormones that have predominantly been measured as T3 effects. It is the opinion of this reviewer that there are four iodothyronines that have significant but not identical biological activities and these are T4, T3, rT3 and 3,5-T2 (see Fig. 1).

The other problem that plagues consideration of literature on thyroid hormone action is that of 'dose'. As cited above, the T3 production rate for the euthyroid rat is approximately 0.25 nmol/100 g/day (approximately 0.16 μg T3/100 g/day). It is common practice for considerably larger doses to be used (I also plead guilty in this respect). For example, 200 μg T3/100 g is sometimes used and justified as being 'receptor saturating'. This is more than 1000 times greater than the replacement dose of T3. Six hours after the intraperitoneal injection of half this amount in the rat, the plasma T3 concentration is 3 μM (Hartong *et al*., 1987). It is not unreasonable to expect that for a significant period of time after the injection of such doses, the plasma T3 concentration is three orders of magnitude above normal euthyroid levels. Such high doses (sometimes in the μM range) are also used in *in vitro* studies. Since free hormone concentrations for a wide variety of vertebrate species are in the pM range (Fig. 2), I have had to assume the results of many studies to be relevant but they may instead turn out to be physiologically unimportant when reexamined at more realistic concentrations.

It is also common practice to give daily injections of thyroid hormones to induce a hyperthyroid condition. Because a single daily bolus will result in a transient peak in plasma concentration it is assumed a large dose is necessary to ensure a high average concentration between the daily injections. In other words, it is assumed that the same amount given as a single daily injection will be less effective than if it was given as smaller multiple injections or by constant infusion. At least in one case, the

opposite is true. The inhibition of TSH secretion by the rat anterior pituitary was substantially more sensitive to the same daily dose of T3 when it was given as twice daily injections than when it was given by constant infusion (Connors & Hedge, 1980). The pulsatile nature of daily injections of T4 or T3 may give misleading information when correlating hormone effects with plasma hormone concentrations as standard practice is often to measure plasma hormone concentration 24 h after the last injection, that is at the trough of the induced large daily cycle in hormone concentration. This point is illustrated by one of the few studies to determine the average daily plasma hormone level following thyroid hormone injection (John-Alder, 1983). In this study, lizards were given daily T4 injections (20 μg/100 g body mass). Whilst control lizards had a total plasma T4 concentration of 3.2 pM, the injected animals had a 24 h post-injection concentration of 12.9 pM, while the 24 h average T4 level was 122 pM. For this reason, it is more desirable to use constant infusion of hormone if at all possible (for example by osmotic minipumps, see Escobar del Rey *et al.*, 1989), unless one is examining the time course of a particular effect following a pulse of hormone.

Another problem of interpretation of results following thyroid hormone injections is consideration of how the treatment alters plasma thyroid hormone levels. Generally, total concentrations of the hormone are measured and it is often not known how the physiologically more relevant free hormone levels are changed by the treatment.

Because of these problems, the hypothyroid-euthyroid comparison is probably more reliable in examining the physiological effects of the thyroid hormones. However, there are also considerations with this comparison. One is that some of the observed effects may be a secondary response rather than a primary effect (of course, this problem is also present in the euthyroid-hyperthyroid comparison). One of the best cases of this problem is the secondary effects of low growth hormone secretion in hypothyroidism and the fact that observed effects of hypothyroidism may be due to growth hormone deficiency rather than thyroid hormone deficiency. Classical hormonal replacement following induced hypothyroidism can resolve some of these problems but once again dose is important.

Cell culture studies can avoid many of these pitfalls but the results of such studies should be verified *in vivo*, especially since many cell lines are originally derived from tumours and thus may not behave normally (e.g. Sorimachi & Robbins, 1977). In a similar vein, many cell culture media whilst being adequate for maintaining a living cell, are not necessarily normal and can thus result in non-physiological conditions. This is especially the case with respect to fatty acid composition of the media and its consequent influence on the membrane lipid composition of some cell cultures.

There is a large reservoir of thyroid hormones in the body, both in the thyroid gland itself and also bound to the plasma proteins. It appears to be very difficult to completely deplete the body of thyroid hormones. Although the plasma T4 and T3 levels decrease relatively rapidly, measurable amounts of the hormones are still present in the tissues of the rat for some time following thyroidectomy (Obregon *et al.*, 1981). In addition, there is evidence that there may be extrathyroidal synthesis of thyroid hormones in the rat following thyroidectomy (Evans *et al.*, 1966).

There are some hormonal effects that can be observed *in vitro* but not *in vivo*. There are two possible reasons for this. The first is that it may be possible to measure particular parameters *in vitro* that are impossible to measure *in vivo*. The second is that the observed effect may be due to some condition that is found *in vitro* but not *in vivo*. Such effects, whilst they may give important insight, might have little relevance to understanding what is happening in the body. However, we should also remind ourselves that much of our knowledge of biochemistry and cell function comes from *in vitro* studies.

Finally, it is worthwhile to remember that both hypothyroidism and hyperthyroidism can be regarded as pathological conditions. Our treatments are artifices we create in order to understand the normal euthyroid state (or of course to understand the pathological state). In the normal (i.e. euthyroid) adult vertebrate, thyroid hormones, and especially their free concentrations, appear generally to remain relatively constant. In the normal state, the thyroid hormone system has both a large reservoir of hormone stored in the gland (it is relatively unusual in this respect) and a large reservoir of easily accessible protein-bound hormone in the blood. Thus, the concept that thyroid hormones regulate a process that normally varies, whilst thyroid hormone levels themselves are relatively constant, suggests that the hormones are not the most important regulator in such situations.

My purpose in the remainder of this review is to attempt to understand the myriad of reported thyroid hormone effects within a few underlying

basic concepts. I have grouped the various effects into categories for the sake of convenience and will first discuss reported effects in adult vertebrates before considering the role of the thyroid in vertebrate development.

(2) Hypotheses regarding thyroid hormone action

Because of the dogmatic nature of the oft-repeated statement that thyroid hormones act *via* nuclear receptors, it is difficult to convince many people that they may not do so. Generally, only some very specific effects have been associated with a non-genomic mode of action. Others have previously questioned the nuclear receptor pathway as the sole mode of action of thyroid hormones (e.g. Segal, 1989*b*, 1990*b*). A recent review of non-genomic actions of thyroid hormones is that of Davis & Davis (1996). Such questioning of a single genomic mode of action for a hormone is not unique for thyroid hormones, as non-genomic actions recently have been proposed for reproductive hormones (Revelli, Massobrio & Tesarik, 1998), and other steroid hormones including aldosterone (Wehling, 1994) as well as for some glucocorticoid effects (Buttgereit, Wehling & Burmester, 1998).

As stated above, there exists evidence that not all thyroid hormone effects are initiated *via* binding of thyroid hormones to nuclear receptors. Some extra-nuclear effects that have been reported have been observed at supra-physiological concentrations of hormone (as indeed are many nuclear-mediated effects) but many have been observed with physiological levels of hormone. Some of the most convincing evidence is that which demonstrates *in vitro* thyroid hormone effects on cells that have no nucleus, that is, on mammalian red blood cells. In human erythrocytes, membrane Ca^{2+}-ATPase activity is stimulated *in vitro* by physiological concentrations of thyroid hormone (Davis, Davis & Blas, 1983) and this has *in vivo* relevance in that erythrocytes from hyperthyroid and hypothyroid humans have, respectively, increased and decreased enzyme activities (Dube *et al*., 1986). Lawrence, Schoenl & Davis (1989) have shown that both T4 and T3 (0.1 nM) stimulated phospholipid-dependent protein kinase activity in rabbit erythrocytes *in vitro*.

There is also evidence that in nucleated cells some thyroid hormone effects cannot be initiated at the level of the nucleus. A very convincing aspect of this evidence is the time course of such effects at physiological hormone concentrations. For example, 1 nM T3 induces an influx of Ca^{2+} into rat thymocytes that is evident within 15 s of hormone administration (Segal & Ingbar 1984). Similarly, application of T3 to GH3 cells in culture (2–5 nl of 0.1 nM T3) resulted in an increase in membrane resistance and a hyperpolarization of the membrane potential. These effects were evident within 1 min of application, and within a few minutes spontaneously firing cells became silent (du Pont & Israel, 1987). These are a few examples of effects which cannot be mediated *via* nuclear receptors.

It is the thesis of the present review that many thyroid hormone effects can be explained by an initial action on cell membranes, not just the plasma membrane but also the other membranes within the cell. I will examine many thyroid hormone effects to see if they can be explained by such an initial site of action, rather than the commonly proposed nuclear-receptor-mediated mode. First, I should state the hypothesis explicitly and say something about membranes. Several years ago I proposed a similar but more rudimentary thesis for such a site of thyroid hormone action (Hulbert, 1978). At that time, I was unaware of an earlier and different proposal for a membrane site of action emphasizing the importance of the iodine atoms of the thyroid hormones (Gruenstein & Wynn, 1970). This earlier proposal was based around many effects of thyroid hormones that are present at supraphysiological levels of hormone and thus may not be relevant to the normal situation. Dratman (1974) proposed that some effects of T4 stemmed from its similarity to (and origin from) tyrosine residues and this proposal has been recently reviewed (Dratman & Gordon, 1996). Several other authors have also argued for a perspective of thyroid hormone action that is broader than the nuclear-receptor hypothesis (e.g. Sterling, 1979; Muller & Seitz, 1984). Hoch (1988) has presented an extensive review of thyroid hormones and lipids which has also argued that many thyroid hormone effects can be explained as a consequence of an effect on membrane fatty acids, although he suggests such membrane changes are due to a nuclear-receptor-mediated action.

The proposal is that the thyroid hormones act in more than one way. One of the ways in which they operate is that they associate with the hydrophobic interior of membrane systems and change the physical behaviour of the lipid component of the membrane. As a consequence of this, the phospholipid and fatty acyl composition of the membrane is altered by normal cellular mechanisms and these changes further alter the physical behaviour of the

lipid bilayer and consequently that of various membrane proteins. For example, due to both the initial direct effects on the bilayer and also the effects of the subsequent fatty acyl changes, there are changes in membrane permeability and the enzymatic activity of membrane-associated systems. These changes may be increases or decreases, and will, in some cases, alter both the distribution and rate of transfer of information between various membrane-limited cellular compartments. Several thyroid hormone effects can be explained by such an initial site of action, especially (but not exclusively) the effects that are related to the stimulation of metabolism. This proposal is unusual in that although these effects may be mediated *via* the binding to some form of 'specific' receptor molecule, they are more likely to come about *via* a less-conventional non-specific mode of action, i.e. not requiring a 'specific' receptor in the normal sense of the word.

Before analyzing the plethora of thyroid hormone effects, I will briefly review both the nuclear receptor mode of action and following that, some aspects of membrane lipids and their function, as well as what we know of thyroid hormone effects on membrane lipid behaviour and composition.

(*a*) *Nuclear receptors and thyroid response elements*

In 1972, specific nuclear binding sites, with limited capacity and high affinity for T3, were found in rat liver and kidney (Oppenheimer *et al.*, 1972). Following this, similar nuclear binding sites were described for a variety of other tissues as well as in various cell cultures. Although they were not isolated and purified, the physico-chemical properties and binding characteristics of these nuclear receptors were defined (for early reviews see DeGroot *et al.*, 1978; Oppenheimer & Samuels, 1983; Oppenheimer *et al.*, 1987; Samuels *et al.*, 1988).

The thyroid nuclear receptor was similar in all tissues and species examined (Oppenheimer *et al.*, 1987). It was a non-histone protein and was most studied in rat liver and in pituitary tumor (GH and GC) cell lines. In rat liver nuclei, it was 50.5 kDa in mass, with approximately 6000 per nucleus and at endogenous T3 levels it was estimated that approximately 50% of nuclear receptors were occupied (Oppenheimer *et al.*, 1974). In GH1 and GC cells, it was 54 kDa in mass, with approximately 15000 per nucleus having an average half-life of approximately 4.5 h (Samuels *et al.*, 1988). The receptor densities in these pituitary tumor cell lines were approximately twice the average found for anterior pituitary *in vivo* (Oppenheimer *et al.*, 1974) and presumably reflected the specific type of anterior pituitary cell from which the cell line was derived. In the rat, receptor density varied between tissues, ranging from approximately 20 per nucleus in the testes to approximately 8000 per average anterior pituitary cell. In GH1 cell cultures, the number of receptors was inversely related to T3 levels (Samuels, Stanley & Shapiro, 1977) whereas in the intact animal similar changes were not observed (Oppenheimer, Schwartz & Surks, 1975; Spindler *et al.*, 1975).

In 1986, partly by accident, the genes for these nuclear proteins were discovered separately by two groups (Sap *et al.*, 1986; Weinberger *et al.*, 1986). The similarity of the viral erb-A oncogene product to the glucocorticoid nuclear receptor suggested that the cellular equivalent of this viral erb-A gene may code for a receptor that bound one of the steroid hormones. However, this proved unsuccessful and it was shown instead to bind thyroid hormones with a similar affinity to the previously described nuclear binding sites, showed the same analog specificities and had a similar molecular weight. Sap *et al.* (1986) had isolated the gene from a library of DNA cloned from the chicken embryo whilst the other group (Weinberger *et al.*, 1986) isolated it from a human placental cDNA library. Since then it has become apparent that there is a superfamily of related transcription control factors that all have both a DNA binding region and another region that binds either a steroid hormone (e.g. glucocorticoid, mineralocorticoid, progesterone, estrogen), or a vitamin (e.g. vitamin D3 and retinoic acid) or the thyroid hormones. Soon after the isolation of the original genes, other cDNA sequences coding for thyroid nuclear receptors were also isolated from rat brain (Thompson *et al.*, 1987), human testis (Benbrook & Pfahl, 1987), human kidney (Nakai *et al.*, 1988) and two from both GH_3 cells (Lazar & Chin, 1988) and rat liver (Murray *et al.*, 1988). The thyroid hormone nuclear receptors are thus members of this superfamily of receptors involved in the control of transcription (for an early review see Evans, 1988).

There are two main types of thyroid hormone receptors, an α and a β form. Both forms are sometimes expressed in the same tissue, and both forms from rat liver bound thyroid hormones with the same affinity and spectrum of analogue binding (Murray *et al.*, 1988). Since the mid 1980s there has been intense research interest in this area, much knowledge has been gained yet the precise mechanism of how the system operates, although slowly

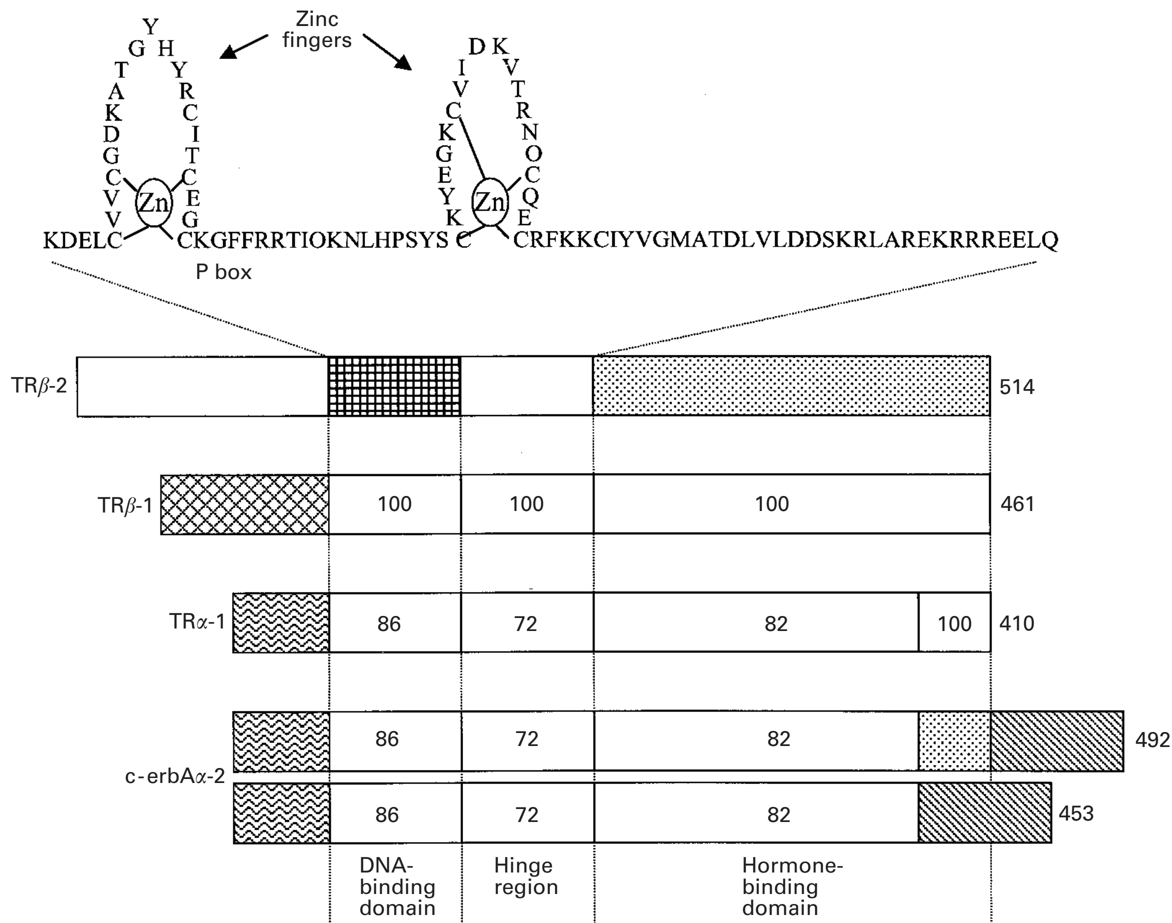

Fig. 8. Domain organization of thyroid nuclear receptors. The three thyroid-hormone-binding receptors (TRα-1, TRβ-1, TRβ-2) and two isoforms of the non-hormone-binding receptor (c-erbAα-2) are shown. The numbers inside the boxes refer to the amino acid sequence % homology of each TR isoform relative to TRβ-2, and the numbers at the end of each box refer to the number of amino acids in each TR isoform. The letters identify individual amino acids. Adapted from figures in Yen & Chin (1994) and Chatterjee *et al.* (1997).

being unravelled, is still elusive. There are several very good reviews to which the reader is referred for more detailed information (Chin, 1991; Martinez & Wahli, 1991; Lazar, 1993; Yen & Chin, 1994; Ribeiro *et al.*, 1995; Oppenheimer, Schwartz & Strait, 1996; Chatterjee, Clifton-Bligh & Matthews, 1997; Zhang & Lazar, 2000). I have generally cited these reviews for information rather than the voluminous primary literature.

In humans, the α gene resides on chromosome 17 and the β gene on chromosome 3. Alternate processing of the initial transcript from each gene yields two isoforms of each receptor. Both isoforms of the β thyroid receptor (TRβ-1 and TRβ-2) but only one of the α receptor isoforms (TRα-1) bind thyroid hormones. The other non-hormone-binding α isoform (c-erbAα-2 although sometimes also called TRα-2) is thus not a true thyroid hormone receptor but may be involved in thyroid hormone effects as it binds to appropriate parts of the genome. All have the same basic structure as other members of the receptor superfamily which as a group are described as ligand-dependent transcription factors. This basic structure is illustrated in Fig. 8. The amino terminal region of the protein is followed by the DNA-binding domain, then a hinge region, followed by the ligand- (i.e. hormone/vitamin) binding domain. The β receptors differ only in their amino terminal region. The α gene products have a shorter amino terminal region than the β receptors and have a high degree of amino acid homology in the DNA-binding region, hinge region and the ligand-binding region (see Fig. 8). The carboxyl terminal region is highly conserved in all the thyroid-hormone-binding forms and is essential for hormone binding. The ligand-binding region of the receptor is composed of many α helices, and X-ray diffraction studies of crystals of the region in TRα-1, both with and without T3 (and ana-

logues) have revealed changes in its three-dimensional structure with hormone binding (McGrath *et al.*, 1994; Wagner *et al.*, 1995). The hormone is located in a deep pocket in this protein with the amino group of the hormone facing the outside. Analysis of the 3-D structure suggests that the receptor adopts different structures to fit the different ligands (i.e. T3 analogues). The conformational change with hormone binding is probably connected to transcriptional activation.

The DNA-binding domain consists of an area relatively conserved between different receptors and it is this region that is responsible for recognizing specific parts of the genome. In thyroid receptors, it contains 68 residues with basic amino acids organized into two 'finger-like' structures that each contain four cysteine residues tetrahedrally coordinated with a zinc atom. It is believed these 'zinc fingers' interact with a half turn of DNA (see Evans, 1988). Also, an amino acid sequence present at the base of the first zinc finger, known as the P box, is important for DNA-binding specificity and serves to divide the receptor superfamily into two groups. The P box is identical in the thyroid hormone receptors (TRs), retinoic acid receptors (RARs), retinoid X receptors (RXRs), vitamin D receptor (VDR) and the peroxisomal-proliferator-activated receptors (PPARs). These receptors preferentially bind to the nucleotide sequence: AGGTCA (Lazar, 1993). Between the DNA-binding and hormone-binding domains, in the hinge region, there is a conserved motif of basic residues that acts as a nuclear localization signal for nuclear pore recognition.

Within the nuclei of isolated rat hepatocytes, TRs are separable into three compartments that are distinguished by the conditions needed for their isolation and thus possibly related to differences in their DNA binding. Nucleoplasmic TRs (45%) can be isolated with isotonic saline, some other TRs (30%) are extracted with high salt (0.4 M KCl) concentrations, whilst the remaining TRs (25%) are salt resistant, requiring high salt and 5 mM dithiothreitol for extraction. Pulse experiments with hepatocytes exposed to extracellular labelled T3 showed that all were rapidly labelled with T3 (within 1 min) with no preference for any particular compartment, that the average occupation time for T3 on a receptor was approximately 3 mins, and that T3 did not influence any shift in receptors between these compartments (Pullen *et al.*, 1994). The sum of these receptors represented 0.2 pmol TR mg^{-1} DNA, which is below the normal 0.5–1.0 pmol mg^{-1} DNA reported for liver homogenates but is consistent with the loss of receptors that has been observed when isolated liver cells are cultured without protection of sulfhydryl groups (Yamamoto *et al.*, 1992).

Using antibodies specific to the particular TR isoforms (which thus interfere with their T3 binding), Schwartz, Lazar & Oppenheimer (1994) were able to show that all three receptor types are widespread in rat tissues. In liver, the receptor concentrations (in pmol mg^{-1} DNA) were 0.71, 0.17, and 0.13 for TRβ-1, TRβ-2 and TRα-1, respectively. The respective values for kidney TRs were 0.10, 0.04 and 0.10 pmol mg^{-1} DNA; for heart they were 0.24, 0.10, and 0.26 pmol mg^{-1} DNA, and for brain cortex they were 0.19, 0.07 and 0.40 pmol mg^{-1} DNA (Schwartz *et al.*, 1994). Not all cells within a tissue, however, have TRs. For example, within the brain, immunofluorescence studies have shown that oligodendrocytes possess all three receptor isoforms in their nuclei but that astrocytes have none (Carlson *et al.*, 1994). However, when astrocytes are examined in cell culture, they do possess TRs (Carlson *et al.*, 1996).

The mRNA for the different TR isoforms varies between tissues but not in relation to their respective TR content. Between tissues the receptor/mRNA ratio can vary by as much as 15-fold (Oppenheimer, Schwartz & Strait, 1996).

In 1986, the year that the TR genes were identified, it was found that thyroid hormone nuclear receptors bound to a particular part of the promoter regions of the human growth hormone gene and the human placental lactogen gene (Barlow *et al.*, 1986). For TRs to activate transcription they must bind to sections of DNA known as 'thyroid response elements' (TREs). The DNA-binding region of the TRs (and some other receptors) has a P box which recognizes the hexanucleotide AGGTCA (Umesono & Evans, 1989).

In the early 1990s it was recognised that TRs could bind to DNA as either monomers or dimers, and that dimers could consist of either the same or different TR isoforms (i.e. either homodimers or heterodimers). It was also discovered that TRs could form heterodimers with an accessory protein, that augmented TR binding to the genome and was later identified as the retinoid X receptor (RXR). It is now appreciated that the RXR–TR dimer shows the strongest binding to the genome. Dimerization is mediated by at least two protein-protein interactions between the adjacent monomers. One interaction region is in the DNA-binding region whilst another is a heptad towards the carboxyl end of the hormone-

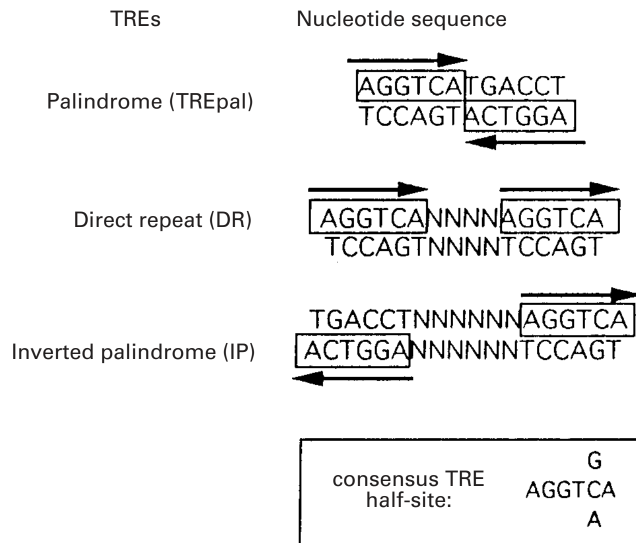

Fig. 9. The nucleotide sequences for thyroid response elements (TREs) involved in the binding of thyroid nuclear receptors to the genome. Reprinted with permission from Yen & Chin (1994).

binding domain of the receptor. In addition, in dimers both receptors bind to the DNA. This has led to the recognition that AGGTCA is thus a half-site for binding of dimers and that TREs for dimers exists as either a direct repeat separated by four bases (called DR+4), an inverted palindrome (TREpal), or an inverted palindrome separated by six bases (IP). These TREs are shown in Fig. 9. When bound as a RXR-TR heterodimer, the TR is bound to the 3′ recognition half-site and it appears that the RXR is not ligand sensitive, indeed the TR appears to block the binding of ligands to RXR upon dimerization (Glass, 1996).

Thyroid receptors are known to bind to TREs both with and without bound thyroid hormone. Dimers generally repress transcription of target genes in the absence of bound hormone and activate transcription when hormone is bound. The precise mechanism of this transcriptional activation is not known but it appears that in the N terminal region there is a weak transcription activation function (AF-1) whilst in the C terminus of the ligand-binding domain there is a more powerful ligand-dependent activation function (AF-2). Various experiments suggest that in the absence of T3, TR-TR homodimers or RXR-TR heterodimers are bound to TREs and that basal transcription is inhibited by intervening co-repressors between the receptors and the transcription initiation complex. Upon T3 binding, TR-TR homodimers dissociate and co-repressors are released whilst in the case of heterodimers both receptors stay attached to the DNA but co-activators replace the co-repressors. In both cases, transcription of the target gene is commenced by allowing the transcription initiation complex to commence transcription (Chatterjee *et al.*, 1997).

The response elements cited above are all positive TREs, in that they are involved in hormone-stimulated transcription activation. Thyroid nuclear receptors can also exhibit ligand-dependant transcription inhibition but how this occurs is poorly understood. Such inhibition may be mediated by TRs binding as monomers to negative TREs which appear to be half-sites not organized in any particular way, although TR-RXR heterodimers may also be involved (Takeda *et al.*, 1997).

Some mutant TRs (either natural or laboratory created) have been shown to have a dominant negative activity, i.e. the ability to inhibit T3-dependent TR-mediated transcriptional activation. In the syndrome of thyroid hormone resistance mutant TRβ have this ability, as does a mutant TRα-1 isolated from patients with hepatocellular carcinoma (K. H. Lin *et al.*, 1997), and c-erbAα-2 (Lazar, 1993). Several studies have also shown that phosphorylation of thyroid nuclear receptors can increase binding, dimerization and transcriptional activity (e.g Lin, Ashizawa & Cheng, 1992; Bhat, Ashizawa & Cheng, 1994; Sugawara *et al.*, 1994).

The majority of experiments over the last several years that have led to the uncovering of the mechanism of thyroid nuclear receptor operation outlined above, have been carried out using artificially constructed systems. Often the TRs have been expressed in bacteria, the TRE has been taken from another source and been transiently transfected, together with a distinct reporter gene, into a cell culture system or combined in an *in vitro* system. Sometimes the cell culture used doesn't normally express TRs and a particular TR gene has been transfected into the cells. Indeed, this is sometimes regarded as necessary in that it allows more precise control of the experimental system. Whilst these sophisticated techniques have been powerful in elucidating the complex mechanisms whereby the thyroid nuclear receptor system may work, they do not necessarily give insight into the physiological importance of the nuclear mode of action in explaining normal thyroid hormone effects in the vertebrate body. Indeed, the finding that expression of nuclear receptors in cell culture systems sometimes differs from the *in vivo* situation means that care should be taken with interpretation and extrapolation of results obtained from such systems.

The finding of increased levels of mRNA for a

particular protein after changes in thyroid status is often presented as evidence that the thyroid hormones are acting *via* thyroid nuclear receptors stimulating transcription. However, this may not necessarily be so, as the level of mRNA is dependent on its rate of production (i.e. transcription) and its rate of disappearance. Even the finding that transcription is stimulated by changes in thyroid status is not necessarily evidence that the thyroid hormones are acting *via* nuclear receptors. In some cases, an equally valid interpretation may be that these hormones are acting pre-transcriptionally, by influencing the rate of information flow to the nucleus and it is this changed information flow that is responsible for the change in the rate of transcription. An evidential requirement for a nuclear mode of action for a thyroid hormone effect is the demonstration of a TRE for the particular gene involved.

The finding of a nuclear receptor for any particular molecule does not exclude another mode of action. For example, retinoic acid as well as acting through a nuclear receptor can also have effects on human erythrocytes, cells which lack nuclei (Smith, Davis & Davis, 1989) and is also known to inhibit binding of T4 to human TTR (Smith *et al.*, 1994).

(*b*) *Thyroid hormones and the membrane bilayer*

Just as DNA has been described as an 'eternal molecule', membranes can be thought of as an 'eternal structure' and are assemblies of proteins and lipid molecules held together by non-covalent bonds. The lipids in membranes include phospholipids and glycolipids as well as cholesterol and sterols, and at least in vertebrates, it is my assertion that cellular membranes normally also contain thyroid hormones. All membrane lipids are amphipathic molecules.

Fatty acyl chains form the hydrophobic section of the phospholipid molecule and the core of the membrane bilayer. Saturated acyl chains are flexible because of the possible rotation around the C–C bonds that form the backbone of the acyl chain The double bonds of unsaturated acyl chains, on the other hand, do not allow rotation around the C=C bond. Monounsaturates have a single C=C unit, whilst polyunsaturates have from two to six such units. The majority of fatty acids in the cellular membranes of vertebrates are between 16 and 22 carbons long and the average chain length is generally around 18 carbons long. In this review, I will refer to specific acyl chains using a numbering system. Thus, stearic acid, a saturated 18 carbon fatty acid will be called 18:0, oleic acid which is an 18 carbon monounsaturate will be referred to as 18:1, whilst docosahexanoic acid a 22 carbon polyunsaturate with six double bonds will be called 22:6. In higher animals, newly synthesised fatty acids are saturated (predominantly 16:0 and 18:0) and some are converted to unsaturated fatty acids by desaturases, which are membrane-bound multi-enzyme complexes that consume oxygen, insert double bonds at specific places in the fatty acyl chain and are named accordingly. The cells of vertebrates have $\Delta 5$, $\Delta 6$, and $\Delta 9$ desaturases but lack $\Delta 12$ or $\Delta 15$ desaturases. The products of the $\Delta 12$ desaturase are called the n-6 (or omega-6) polyunsaturates, whilst the products of the $\Delta 12$ desaturase that have been further processed by the $\Delta 15$ desaturase are known as the n-3 (or omega-3) polyunsaturates. The average membrane bilayer consists of two 18 carbon thick monolayers. Membranes without polyunsaturates will have no C=C units in the middle half of membrane bilayer whilst membranes with only n-6 polyunsaturates will have no C=C units in the middle third of the bilayer. It is only the n-3 acyl chains that can contribute C=C units to the middle portion of membranes. The unsaturated fatty acids that vertebrates generally synthesize *de novo* are the n-9 or n-7 monounsaturates which will contribute C=C units to bilayers in two regions both approximately one-quarter of the way into the bilayer from each side.

In 1972, Singer & Nicholson proposed the 'fluid mosaic' model of membrane structure and although it has since been modified and extended, it is still the basis of modern thinking concerning cellular membranes. Over the last three decades, it has become apparent that the hydrophobic compartments of the cell (i.e. its membranes) are a series of complex microenvironments that influence many aspects of cellular function. It has also become apparent in the last quarter of a century that these aspects of membrane structure are regulated, in that there are homeostatic systems within the cell to maintain an appropriate 'fluidity' (or viscosity) of the membrane bilayer when conditions act to change membrane 'fluidity'. One of the most obvious of these environmental factors is temperature but there are several others. Following its discovery in bacteria, this response was termed 'homeoviscous adaptation' (Sinensky, 1974). It is possibly one of the earliest homeostatic responses evolved by cells and has been shown to also be present in both plants (Thompson & Nozawa, 1984) and animals (Cossins, Bowler & Prosser, 1981). Although it involves a number of

responses, this self regulation of membrane 'fluidity' is mainly brought about by manipulation of membrane fatty acyl composition, particularly the degree of unsaturation of membrane fatty acyl chains. It thus involves the lipid desaturases. In a wide variety of eukaryotic cells, it has been shown that the activity of the lipid desaturases is inversely affected by the fluidity of the membrane with which they are associated and it has been suggested that this relationship is one of the mechanisms of the regulation of membrane fluidity (Brenner, 1981; Kates, Pugh & Ferrante, 1984; Thompson & Nozawa, 1984). Accordingly, a decrease in membrane fluidity (or an increase in membrane rigidity), whether it be caused by a decrease in temperature, or the increased membrane incorporation of saturated fatty acids or of sterols (e.g. Leikin & Brenner, 1989) or by other means (such as the experimental incorporation of unnatural 'rigidizers' etc.) will result in stimulation of the desaturase complexes and a consequent increase in the degree of unsaturation of membrane fatty acyl chains. As in any homeostatic system the converse is also true. This is presumably why although changes in the fatty acyl composition of the diet may influence the relative occurrence of individual acyl chains in membrane lipids it generally does not significantly affect the overall degree of unsaturation of the membranes (e.g. Withers & Hulbert, 1987). Diet can, however, have very significant effects by influencing the balance between the different types of polyunsaturates in membranes (e.g. Pan, Hulbert & Storlien, 1994).

This process is not a perfect homeostatic response and degrees of homeostasis have been observed. For example the sarcoplasmic reticulum exhibits a lack of 'homeoviscous adaptation' (Cossins, Christiansen & Prosser, 1978). Although such responses consist predominantly of changes in acyl chain composition they also include other alterations such as changes in the ratio of different phospholipid classes. The mechanisms involved include *de novo* synthesis of phospholipids and direct desaturation of membrane acyl chains but predominantly the membrane remodelling involves a cycle of deacylation/reacylation of membrane phospholipids which is accomplished by the combined action of two types of enzymes: phospholipases and lysophospholipid acyltransferases (Hazel & Williams, 1990). Indeed, although there are hundreds of different molecular species of phospholipids in membranes, in rat hepatocytes only four molecular phospholipid species are synthesised *de novo* and the diversity of membrane phospholipid species is due to the remodelling of these four initial phospholipid species by deacylation/reacylation processes (Schmid, Deli & Schmid, 1995).

I have spent some time describing membrane lipids and have concentrated on the role of acyl chains because it is important in the consideration of my argument concerning the effects of thyroid hormones. Our understanding of the roles of membrane acyl composition in influencing many different membrane-associated processes is increasing and has been reviewed elsewhere by others (Brenner, 1984; Stubbs & Smith, 1984; Spector & Yorek, 1985; Bernardier, 1988; Murphy, 1990). Because of the need to concentrate on the role of the fatty acyl residues in membrane function in the present review, discussion of membrane bilayer function has by necessity been simplified, omitting for example, consideration of such things as the asymmetry of exofacial and cytofacial leaflets of membranes, as well as important considerations of phospholipid head groups. The potential importance of membrane acyl composition and especially the degree of polyunsaturation of membrane bilayers on the cellular metabolic activity of vertebrates has been recently discussed by Hulbert & Else (1999, 2000).

Because of the amphipathic nature of the thyroid hormone molecules that have a non-ionized phenolic −OH, they will be found in the membrane bilayer in high concentrations and oriented with the phenolic end of the molecule towards the centre of the bilayer. Would their presence affect the physical behaviour of membrane lipids? Dickson *et al.* (1987) have shown that both T4 and T3 quench the light emitted by fluorescent groups inserted in artificial phosphatidylcholine bilayers. The pH characteristics of this quenching suggest that T4 and T3 molecules with the non-ionized phenolic −OH are responsible for this quenching. Farias *et al.* (1995) have also measured the effects of T3 and T4 on liposome bilayers and have shown that both thyroid hormones 'rigidify' membranes in the liquid-crystalline phase, that L- and D-isomers have the same effect but that T3 differs from T4 in that when membrane bilayers are in a gel state (i.e. below their phase transition temperature) T3 is also capable of 'fluidizing' the liposomal membrane. In this respect, T3 is similar to cholesterol. The presence of cholesterol in liposome bilayers also influences the effects of both T4 and T3. In the absence of cholesterol, T3 and T4 were incorporated into the liposome at respectively 9 and 6 nmol of hormone per 100 nmol of phospholipid. As cholesterol content of liposomes increases a decreased amount of both

T4 and T3 is incorporated into the liposome membrane. The influence of both T3 and T4 on membrane fluidity and membrane permeability (both assessed by fluorescent probes) is also dependent on the cholesterol content of the liposomal membrane (Chehin *et al.*, 1995). Although in this study the T4 and T3 concentrations used for measurement of both leakage and fluorescence polarization were in the μM range, and are thus supraphysiological, the incorporation measurements were performed at physiological thyroid hormone concentrations. Incorporation of 50% mol cholesterol into liposome membranes decreased T3 incorporation by 76% and T4 incorporation by 99 % compared to cholesterol-free liposomes (Chehin *et al.*, 1995).

An early and significant study is that by Schroeder (1982) who showed that T3 *in vitro* resulted in rigidification of the rat liver plasma membrane bilayer. These results, because they occurred immediately cannot be due to other thyroid-induced changes in membrane lipid composition. They occurred at physiological concentrations and also occurred in a natural membrane. They were measured using two separate membrane fluorophores and the results show the same response as measured by others on artificial membranes (unfortunately often at higher T3 concentrations). It is tempting to speculate that thyroid hormones 'rigidify' the membrane bilayer because the large electronegative iodine atoms in the thyroid hormones induce increased van der Waals forces in the surrounding acyl chains and thereby diminish their mobilities. Liver plasma membranes isolated from hyperthyroid rats (T3 injections), euthyroid controls and hypothyroid rats had a similar fluidity in the absence of Ca^{2+} ions (Schroeder, 1982).

In an *in vivo* context, Beleznai *et al.* (1989) have shown that both liver mitochondrial membranes and mitoplasts from hypothyroid rats are less rigid, whilst those from hyperthyroid rats are more rigid than those from euthyroid rats. Their use of both DPH (diphenylhexa-1,3,5-triene) and TMA-DPH (1-[4-trimethyl-aminophenyl-phenyl]-6-phenylhexa-1,3,5-triene) which, respectively, associate with the inner hydrophobic core and the outer area of membranes showed that both parts of the bilayer were similarly affected. Similarly, Parmar *et al.* (1995) have shown that liver mitochondria from thyroidectomized rats have less rigid membranes than euthyroid controls and that replacement injections of T4, at physiological levels, resulted in a rigidification of these mitochondrial membranes. Brasitus & Dudeja (1988) have shown that the apical plasma membranes of rat colon are more fluid (less rigid) in hypothyroid rats than in euthyroid controls. T4 injections into rabbits resulted in muscle plasma membranes that were more rigid than those from control rabbits (Pilarski *et al.*, 1991).

Both T4 and T3 injections into euthyroid rats are reported to have an opposite effect on brain mitochondria in that they resulted in an increase in fluidity (a decrease in rigidity); T4 was slightly more potent at the concentrations used than was T3 (Bangur, Howland & Katyare, 1995). When the fluidity of different membrane fractions from the brain of hypothyroid rats was compared with euthyroid controls a significant increase in microviscosity was measured in the mitochondrial fraction but not in the microsomal, synaptosomal or myelin fraction (Tacconi *et al.*, 1991).

In these *in vivo* studies cited on hyperthyroid or hypothyroid mammals, there were also significant thyroid-status-related changes in membrane lipid composition (including phospholipid headgroup composition, cholesterol content, and fatty acyl composition). Many of these changes themselves influence membrane fluidity and thus it is not always possible to relate the reported *in vivo* changes in membrane fluidity/rigidity solely to direct effects of the thyroid hormones.

Can thyroid-hormone-induced changes in membrane fluidity/rigidity affect the behaviour of proteins located in the lipid bilayer? The Hill coefficient of a reaction can be used to measure small changes in enzyme-membrane interactions. Physiological concentrations of T3 *in vitro* have been shown to change significantly the Hill coefficient for the allosteric inhibition of two different enzyme systems in erythrocyte membranes from rats (De Mendoza *et al.*, 1977). This study provides evidence that T3 decreases membrane fluidity and also illustrates three important points. Firstly, thyroid-hormone-induced changes in membrane fluidity can affect different membrane-bound enzyme systems within the same membrane differently. For example, T3 increased the Hill coefficient for erythrocyte Na^{+}, K^{+}-ATPase activity whilst it decreased it for the erythrocyte acetylcholinesterase. Secondly, such changes in membrane fluidity are general effects in that they can be demonstrated in non-vertebrates: T3 also decreased the Hill coefficient for the allosteric inhibition of Ca^{2+}-ATPase in bacterial membranes. Thirdly, the influence of thyroid hormones on such membrane systems depends on the original fatty acyl composition of the membrane.

Table 2. *The influence of hypothyroidism on membrane fatty acyl composition in mammals*

Species	Tissue	Membrane	Lipid class	Fatty acyl chain 16:0	18:0	18:1	18:2	20:4	22:6	20:4/18:2	Comments	Reference
Rat	Liver	Mitochondria	TL	0	–	–	+	–				Patton & Platner (1970)
Rat	Liver	Mitochondria	TL	0	0	0	+	0				Withers & Hulbert (1987)
Rat	Liver	Mitochondria	PL	+	0	–	+	–		–		Chen & Hoch (1977)
Rat	Liver	Mitochondria	PC	0	0	0	+	–		–		Hoch *et al*. (1981)
Rat	Liver	Mitochondria	PE	0	–	0	+	–		–		Hoch *et al*. (1981)
Rat	Liver	Mitochondria	CL	0	0	0	0	0		0		Hoch *et al*. (1981)
Rat	Liver	Mitochondria	PS	0	0	0	0	0		–		Hoch *et al*. (1981)
Rat	Liver	Mitochondria	PL	0	0	+	+	–		–		Hoch *et al*. (1981)
Rat	Liver	Mitochondria	TL	0	0	0	+	–		–		Ismailkhodzhaeva *et al*. (1986)
Rat	Liver	Mitochondria	PC	+	0	0	+	–	–	–		Raederstorff *et al*. (1991)
Rat	Liver	Mitochondria	PE	0	0	+	+	–	–	–		Raederstorff *et al*. (1991)
Rat	Liver	Mitochondria	TL	0	0	0	+	–		–		Paradies *et al*. (1991)
Rat	Liver	Microsomes	PL	0	0	0	+	–		–		Hoch *et al*. (1981)
Rat	Liver	Microsomes	TL	0	0	0	+	–		–		Faas & Carter (1982)
Rat	Liver	Microsomes	PC	0	0	0	+	–		–		Faas & Carter (1982)
Rat	Liver	Microsomes	PE	0	0	0	+	–		–		Faas & Carter (1982)
Rat	Liver	Microsomes	PS/PI	0	0	0	+	0		–		Faas & Carter (1982)
Rat	Liver	Microsomes	PL	0	0	0	+	–	0	–		Tacconi *et al*. (1991)
Rat	Liver	Nuclei	PL	0	–	+	+	–		–		Shaw & Hoch (1976)
Rat	Heart	Mitochondria	TL	0	0	0	0	0				Steffen & Platner (1976)
Rat	Heart	Microsomes	TL	0	0	0	0	0				Steffen & Platner (1976)
Rat	Heart	Mitochondria	PL	0	–	–	+	0		–		Shaw & Hoch (1977)
Rat	Heart	Mitochondria	PL	0	0	0	+	–		–		Hoch (1982)
Rat	Heart	Mitochondria	PC	0	–	0	+	–	+	–	n-6 diet	Pehowich (1995)
Rat	Heart	Mitochondria	PC	0	–	0	+	–	0	–	n-3 diet	Pehowich (1995)
Rat	Heart	Mitochondria	PE	0	–	+	+	+	0	–	n-6 diet	Pehowich (1995)
Rat	Heart	Mitochondria	PE	0	–	+	+	0	0	–	n-3 diet	Pehowich (1995)
Rat	Heart	Mitochondria	CL	0	+	–	0	0	0	0	n-6 diet	Pehowich (1995)
Rat	Heart	Mitochondria	CL	–	–	–	+	–	0	–	n-3 diet	Pehowich (1995)

Rat	Heart	Mitochondria	TL	+	−	0	−	+	0	+		Paradies & Ruggiero (1989)
Rat	Heart	Sarcolemma	PC	0	0	−	+	0	0	−	n-6 diet	Pehowich & Awumey (1995)
Rat	Heart	Sarcolemma	PE	+	−	+	+	+	0	−	n-6 diet	Pehowich & Awumey (1995)
Rat	Heart	Sarcolemma	PC	0	0	+	0	0	0	−	n-3 diet	Pehowich & Awumey (1995)
Rat	Heart	Sarcolemma	PE	+	−	+	0	−	0	−	n-3 diet	Pehowich & Awumey (1995)
Rat	Muscle	Sarcoplasmic reticulum	PC	−	+	−	−	+				Simonides & van Hardeveld (1987)
Rat	Muscle	Sarcoplasmic reticulum	PE	−	0	−	−	+				Simonides & van Hardeveld (1987)
Rat	Brain	Microsomes	PL	0	0	0	0	0	0			Tacconi *et al*. (1991)
Rat	Brain	Mitochondria	PC	0	0	0	0	0	0			Tacconi *et al*. (1991)
Rat	Brain	Mitochondria	PE	0	0	0	0	0	0			Tacconi *et al*. (1991)
Rat	Brain	Synaptosomes	PC	0	0	0	0	0	0			Tacconi *et al*. (1991)
Rat	Brain	Synaptosomes	PE	0	0	0	0	0	0			Tacconi *et al*. (1991)
Rat	Brain	Myelin	PC	0	0	0	0	0	0			Tacconi *et al*. (1991)
Rat	Brain	Myelin	PE	0	0	0	0	0	0			Tacconi *et al*. (1991)
Rat	Colon	Plasma membrane	TL	0	0	0	+	−				Brasitus & Dudeja (1988)
Rat	Erythrocyte	Membrane	PL	0	0	0	0	−	0	−		Tacconi *et al*. (1991)
Rat	Erythrocyte	Membrane	PC	0	0	0	+	−	0			Tacconi *et al*. (1991)
Rat	Erythrocyte	Membrane	PE	0	0	0	+	−	0			Tacconi *et al*. (1991)
Rat	Plasma		PL	0	0	0	+	−	0			Tacconi *et al*. (1991)
Rabbit	Heart	Sarcolemma	PL	+	+	−	0	+		+		Syzmanska *et al*. (1991)
Human	Leucocyte	Plasma membrane	PL	0	0	0	+	0				Van Doormaal *et al*. (1986)
Human	Erythrocyte	Plasma membrane	PL	0	0	0	+	0				Van Doormaal *et al*. (1986)
Human	Platelet	Plasma membrane	PL	0	0	0	+	−				Valdemarsson & Gustafson (1988)
Human	Plasma		PL	0	0	+	+	−				Valdemarsson & Gustafson (1988)
Human	Plasma		PL	−	−	0	+	−				Van Doormaal *et al*. (1986)
Human	Plasma		PL	0	0	0	0	−				Kirkeby (1972*b*)

TL, total lipid; PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin.
+, statistically significant increase; −, statistically significant decrease; 0, no significant effect.
In some experiments, diets with different fatty acyl composition were examined.

From the previous discussion of homeoviscous adaptation of membranes, we would predict that a thyroid-hormone-induced 'rigidifying' effect on membranes should stimulate a change in membrane fatty acyl composition to compensate for the change in membrane physical state. Over the last three decades, many studies have shown that membrane fatty acyl composition is influenced by thyroid status. I have collated these studies into two groups and summarized their findings. Table 2 summarizes the findings showing membrane fatty acyl composition changes induced by hypothyroidism in mammals, whilst Table 3 shows the effects of thyroid hormone injections on the membrane fatty acyl composition of mammals. Most of these studies have not been published with a view to addressing the hypothesis stated above, but rather have been concerned with thyroid-induced changes in lipid metabolism. The two Tables have been restricted to studies examining the fatty acyl composition of phospholipids or, in some cases, the composition of total lipids where the total lipids are predominantly membrane lipids. Only statistically significant effects are shown. The species include humans, rabbits and rats. The tissues include the liver, heart, intestine, skeletal muscle, brain, erythrocytes and leucocytes. The subcellular membranes include those from mitochondria, microsomes, sarcoplasmic reticulum, nuclei, and plasma membranes. In some studies, individual phospholipid classes were also analyzed for changes in their acyl composition.

The diversity of animal treatments, techniques and original purposes for the varied studies precludes a detailed analysis. For example, the range of thyroid hormone injection treatments is huge, ranging from some that are mildly hyperthyroid to some that are thyrotoxic. However, we can conclude that almost every membrane system examined showed a change in its fatty acyl composition with the most consistent change (the few exceptions are discussed below) being an increase in the 18:2 content of membranes with hypothyroidism and a decrease with hyperthyroidism. In addition, there was generally a reciprocal change in membrane 20:4 content, namely a decrease in hypothyroidism and an increase following thyroid hormone injection. Many of these studies have only reported the main fatty acyl chains, and in many the influence of thyroid hormones on n-3 polyunsaturates were not measured.

The only study to examine thyroid-hormone-induced changes in acyl composition of brain phospholipids found only small changes which were not statistically significant, but were in the same direction as found for other tissues, namely hypothyroidism resulted in an increase in 18:2 and a decrease in 20:4 (Tacconi *et al.*, 1991).

One of the exceptions to this finding is the results for sarcoplasmic reticulum (Simonides & van Hardeveld, 1987). It is of interest that the sarcoplasmic reticulum is one membrane that is reported not to exhibit homeoviscous adaptation (Cossins *et al.*, 1978). Two other exceptions either do not agree with other studies for the same membrane and are for total lipids rather than phospholipids (Paradies & Ruggiero, 1989) or are for thyrotoxic rabbits (Pilarski *et al.*, 1991).

Membrane fatty acid content is not the only variable that influences membrane fluidity. For example, cholesterol also acts to decrease membrane fluidity. Cholesterol is normally not a large component of intracellular membranes but is an important constituent of plasma membranes. In mammalian tissues, the cholesterol/phospholipid ratio is approximately 0.1–0.2 for mitochondria and endoplasmic reticulum but is approximately 0.8 for the plasma membrane (Schroeder, Wood & Kier, 1998). Although thyroid status has dramatic effects on plasma cholesterol levels (hypothyroidism is associated with serum hypercholesterolemia and *vice versa*, see Heimberg, Olubadewo & Wilcox, 1985), it appears not to have the same effects on membrane cholesterol levels. In rats, hyperthyroidism resulted in an increase in both cholesterol and phospholipid content of erythrocyte membranes but no change in the cholesterol:phospholipid ratio, whilst in hypothyroidism the decrease in phospholipid content was greater than the decrease in cholesterol content, with a consequent small increase in the cholesterol: phospholipid ratio (Digiorgio, Cafagna & Ruggiero, 1988). Hypothyroidism had no significant effect on the cholesterol content of rat liver microsomes (Faas & Carter, 1982), sarcoplasmic reticulum from rat fast muscle (Simonides & van Hardeveld, 1987), or human erythrocytes (van Doormal *et al.*, 1986) although it did result in a significant decrease in the cholesterol content of rat colonic apical plasma membranes (Brasitus & Dudeja, 1988). Hyperthyroidism had no significant effect on the cholesterol content of rat liver microsomes (Faas & Carter, 1981; Ruggiero *et al.*, 1984) but resulted in a significant decrease in cholesterol content in rat fast muscle sarcoplasmic reticulum (Simonides & van Hardeveld, 1987) and liver mitochondria (Ruggiero *et al.*, 1984). The results concerning the cholesterol content (relative to fatty acid content) of heart

mitochondria are contradictory, one study suggesting that thyroid hormones significantly decrease the cholesterol content (Clejan *et al.*, 1981) whilst another suggests there is no effect (Hoch, 1982). The influence of thyroid status on membrane cholesterol content is not as consistent as that on membrane fatty acid profile but should be taken into account as the presence of cholesterol (and other sterols) in membranes influences membrane desaturase activities both *in vivo* and *in vitro* (e.g. Leikin & Brenner, 1989).

As well as thyroid status affecting the level of plasma cholesterol it also influences the composition of plasma fatty acids. In hyperthyroid humans (and in a euthyroid individual after a single intake of T4), the 18:2 content of plasma cholesterol esters, plasma phospholipids and plasma triglycerides is significantly reduced (Kirkeby, 1972*a*,*b*). Conversely, hypothyroidism results in a statistically non-significant increase in the 18:2 content and a significant decrease in the 20:4 content of plasma phospholipids (Kirkeby, 1972*b*; see Table 2). These changes in plasma lipids are the same as those observed for membranes and it is possible that these plasma changes are the result of changes at the level of the membrane Δ6 desaturase.

In view of the major mechanisms regulating membrane acyl composition, namely the deacylation/reacylation cycle, it is of interest that T4 has recently been reported to stimulate the acylation of lysophosphatidylethanolamine in rat heart (Dolinsky & Hatch, 1998). Similarly, in rats, hypothyroidism results in a decrease in the activities of liver mitochondrial phospholipase A_2 and cytosolic lysophospholipase and an increased activity of two liver microsomal acyltransferase enzymes (Dang, Faas & Carter, 1985). The means whereby the activities of these enzymes are altered due to thyroid status are not known. That they are responsive to changes in membrane properties and are part of the system regulating membrane acyl composition is illustrated by the finding that changes in its surrounding lipid environment can influence the affinity of a rat liver microsomal acyltransferase for its acyl-CoA substrates (Fyrst *et al.*, 1996).

Many of the studies listed in Tables 2 and 3 demonstrated reciprocal changes in 18:2 and 20:4 content. The Δ6 desaturase is the important step for the conversion of 18:2 to 20:4 and thus there is indirect evidence that the activity of this enzyme is decreased in the hypothyroid state and increased by the presence of thyroid hormones. Direct measurement of Δ6 desaturase activity in rat liver microsomes has shown that it is significantly decreased in hypothyroidism (Faas & Carter, 1982). However, the same authors in the previous year (Faas & Carter, 1981) reported a significant decrease in Δ6 desaturase activity following T3 injections in normal rats. These doses (two orders of magnitude greater than the euthyroid rate of T3 production) are possibly too high to give any physiological relevance to this result. Curiously, the same study found that although measured Δ6 desaturase activity was reduced, the 18:2 content of the liver microsomes was significantly decreased and the 20:4 content significantly increased, which is the opposite of what one would expect from the reported changes in enzyme activity. In addition, they found the same dose to result in a very significant increase in Δ6 desaturase activity in food-restricted rats (Faas & Carter, 1981).

Hoch (1988) also reported that hypothyroidism results in a significant decrease in Δ6 desaturase activity and T3 injection into hypothyroids results in a significant increase in enzyme activity but thyrotoxic levels of T3 result in a decreased enzyme activity. Similarly de Gomez Dumm, de Alaniz & Brenner (1977) also showed a decrease in Δ6 desaturase activity at grossly thyrotoxic levels of T4 injections. This illustrates once again the need to use physiologically relevant doses of hormones in experimental situations.

Are these thyroid-hormone-induced changes in membrane fatty acid composition mediated by a nuclear or an extra-nuclear site of action? Some insight into this could be gathered from the time course of such changes. Hoch (1988, p. 204) reports that within 1–2.5 h after T3 injection into hypothyroid rats there were significant changes in membrane fatty acid composition of rat liver mitochondria (specifically, decreases in 18:2, 20:4, and 22:6, with increases in 16:0 and 18:0). These changes had begun by 0.5 h after the T3 injection, but were not statistically significant at that time; however, there were only two animals in the 0.5 h sample. He also reports (Hoch, 1988, p. 221) that within 1 h after the T3 injection there was a significant increase in Δ6 desaturase activity in the liver microsomes in these rats. Once again the changes were manifest within 0.5 h after hormone injection but probably because only two animals were examined there was no statistical significance to the difference. These results suggest that the membrane fatty acid changes occur too quickly to be a nuclear-receptor-mediated effect and may occur as a direct effect of the thyroid hormones on the

Table 3. *The influence of thyroid hormone injections on membrane fatty acyl composition in mammals*

Species	Tissue	Membrane	Lipid class	Fatty acyl chain 16:0	18:0	18:1	18:2	20:4	20:4/18:2	Treatment	Reference
Rat	Liver	Mitochondria	TL	–	+	0	–	0		E/T4; 2000 × 10	Patton & Platner (1970)
Rat	Liver	Mitochondria	TL	0	+	0	–	–		E/T4; 2000 × 8	Platner *et al.* (1972)
Rat	Liver	Mitochondria	PL	0	+	0	–	+	0	HX/T4; 5 × 7	Clejan *et al.* (1980)
Rat	Liver	Mitochondria	PL	–	+	0	–	+	+	H/T4; 50 × 1(4)	Chen & Hoch (1977)
Rat	Liver	Mitochondria	PL	–	0	0	–	+	+	E/T3; 30 × 5	Ruggiero *et al.* (1984)
Rat	Liver	Mitochondria	PC	0	+	0	–	+	+	E/T3; 30 × 5	Ruggiero *et al.* (1984)
Rat	Liver	Mitochondria	PE	0	+	–	–	+	+	E/T3; 30 × 5	Ruggiero *et al.* (1984)
Rat	Liver	Mitochondria	PI	+	+	0	–	0	+	E/T3; 30 × 5	Ruggiero *et al.* (1984)
Rat	Liver	Mitochondria	CL	+	+	0	–	+	+	E/T3; 30 × 5	Ruggiero *et al.* (1984)
Rat	Liver	Mitochondria	PL	–	+	0	0	+	+	E/T4; 100 × 5	Ismailkhodzhaeva *et al.* (1986)
Rat	Liver	Mitochondria	PC	0	0	0	–	+	+	H/T4; 0.5 × 2 (oral)	Raederstorff *et al.* (1991)
Rat	Liver	Mitochondria	PE	0	0	0	–	+	+	H/T4; 0.5 × 2 (oral)	Raederstorff *et al.* (1991)
Rat	Liver	Mitochondria	TL	0	+	0	–	+	+	E/T3; 30 × 5	Paradies & Ruggiero (1990*a*)
Rat	Liver	Mitochondria	TL	+	+	0	–	+	+	H/T3; 100 × 1(1.0–2.5)	Hoch (1988)
Rat	Liver	Microsomes	PL	0	0	0	0	0	+	E/T3; 30 × 5	Ruggiero *et al.* (1984)
Rat	Liver	Microsomes	PC	0	+	–	0	0	–	E/T3; 30 × 5	Ruggiero *et al.* (1984)
Rat	Liver	Microsomes	PE	0	+	0	0	0	+	E/T3; 30 × 5	Ruggiero *et al.* (1984)
Rat	Liver	Microsomes	PI	0	0	0	0	0	0	E/T3; 30 × 5	Ruggiero *et al.* (1984)
Rat	Liver	Microsomes	TL	0	0	0	–	0		E/T3; 500 × 5	Faas & Carter (1981)
Rat	Liver	Microsomes	TL	–	+	0	–	+	+	E/T3; 25 × 21	Faas & Carter (1981)
Rat	Liver	Microsomes	TL	–	+	0	–	+	+	E/T3; 25 × 21	Faas & Carter (1981)
Rat	Liver	Microsomes	PL	0	+	–	–	+	+	HX/T4; 5 × 16	Gueraud & Paris (1997)
Rat	Heart	Mitochondria	PL	0	0	0	0	+	+	HX/T4; 5 × 7	Clejan *et al.* (1981)
Rat	Heart	Mitochondria	TL	0	+	0	–	0		E/T4; 100 × 10	Steffen & Platner (1976)
Rat	Heart	Mitochondria	PL	+	0	0	–	0	0	H/T4; 50 × 1(3)	Shaw & Hoch (1977)
Rat	Heart	Mitochondria	PL	0	0	+	0	–	–	H/T3; 25 × 1(1)	Shaw & Hoch (1977)
Rat	Heart	Mitochondria	PL	0	0	+	–	0	0	H/T3; 25 × 1(2)	Shaw & Hoch (1977)
Rat	Heart	Mitochondria	PL	0	0	+	–	0	0	H/T3; 25 × 1(3)	Shaw & Hoch (1977)
Rat	Heart	Mitochondria	PL	0	0	+	0	–	0	E/T3; 100 × 3	Hoch (1982)

Rat	Heart	Mitochondria	PL	−	+	+	−	0	+	E/T3; 30 × 5	Paradies & Ruggiero (1988)
Rat	Heart	Mitochondria	CL	0	0	0	0	0	0	E/T3; 30 × 5	Paradies *et al*. (1994)
Rat	Heart	Microsomes	TL	+	+	0	−	−		E/T4; 100 × 10	Steffen & Platner (1976)
Rat	BAT	Mitochondria	PL	0	0	0	0	0		E/T4; 50 × 20	Ricquier *et al*. (1975)
Rat	BAT	Mitochondria	PL	0	+	−	0	0	0	E/T3; 30 × 7	Ruggiero *et al*. (1989)
Rat	BAT	Mitochondria	PC	0	+	0	0	0	0	E/T3; 30 × 7	Ruggiero *et al*. (1989)
Rat	BAT	Mitochondria	PE	0	0	0	−	+	+	E/T3; 30 × 7	Ruggiero *et al*. (1989)
Rat	BAT	Mitochondria	CL	0	+	−	+	0	0	E/T3; 30 × 7	Ruggiero *et al*. (1989)
Rat	BAT	Microsomes	PL	0	0	0	0	+	+	E/T3; 30 × 7	Ruggiero *et al*. (1989)
Rat	BAT	Microsomes	PC	0	+	0	−	+	+	E/T3; 30 × 7	Ruggiero *et al*. (1989)
Rat	BAT	Microsomes	PE	+	−	+	0	−	−	E/T3; 30 × 7	Ruggiero *et al*. (1989)
Rat	Muscle	Sarcoplasmic reticulum	PC	+	−	+	−	+	+	H/T3; 10 × 7	Simonides & van Hardeveld (1987)
Rat	Muscle	Sarcoplasmic reticulum	PE	+	0	+	+	−	−	H/T3; 10 × 7	Simonides & van Hardeveld (1987)
Rabbit	Muscle	Sarcolemma	PL	0	0	−	0	+		E/T4; 50 × 7	Pilarska *et al*. (1991)
Rabbit	Muscle	Sarcolemma	PC	+	0	−	+	+		E/T4; 50 × 7	Pilarska *et al*. (1991)
Rabbit	Muscle	Sarcolemma	PE	−	+	−	+	+		E/T4; 50 × 7	Pilarska *et al*. (1991)

BAT, brown adipose tissue; TL, total lipid; PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; CL, cardiolipin.

'+', statistically significant increase; '−', statistically significant decrease; 0, no significant effect.

Treatments are given as: initial condition/thyroid hormone injected; μg hormone(per 100 g body mass) × number of days injected (h after injection).

E, euthyroid; H, hypothyroid; HX, hypophysectomized; T4, thyroxine; T3, triiodothyronine.

membrane lipid environment and consequently on Δ6 desaturase activity. It is of interest that within 0.5 h of T3 injection as well as a change in the enzyme activity there was a large change in the Michaelis–Menten constant (K_m) of the Δ6 desaturase enzyme which was present for at least 2.5 h following hormone injection (Hoch, 1988). It is possible that the change in Δ6 desaturase activity is primarily responsible for the membrane fatty acid changes although more work is necessary to ascertain definitely this as the mode of action.

To my knowledge, nothing is known of the effects of other iodothyronines and thyroid hormone analogues on membrane fatty acid composition.

(3) Effects on the thyroid hormone axis

The concentration of thyroid hormones in plasma and tissues is under the control of the thyroid hormone axis which consists of the following: (1) thyrotropin-releasing hormone (TRH) secretion by cells in the hypothalamus, (2) TSH secretion by cells of the anterior pituitary, (3) the plasma binding proteins, (4) cellular uptake mechanisms, (5) intracellular deiodinases, and (6) nuclear thyroid hormone receptors.

In conscious undisturbed sheep, thyroidectomy results in increased TRH secretion and T4 replacement reverses this effect (Dahl *et al*., 1994). This negative influence of T4 on TRH also occurs in the rat (Rondeel *et al*., 1988). Transfection of the promoter region of the human TRH gene, together with thyroid receptor genes into cell cultures has shown that T3 can repress transcription (Hollenburg *et al*., 1995). The latter study identified two structurally different negative TREs (both single half-sites that bind TR monomers) which cooperate to allow negative regulation of the human TRH promoter, and suggested that this may be restricted to TRβ. More recently, it has been shown that the TRβ-2 isoform, which is most expressed in the hypothalamus and anterior pituitary, has a greater effect than either TRβ-1 or TRα-1 and that this effect is mediated by a novel amino terminal domain of the receptor (Langlois *et al*., 1997).

Thyroid hormones are the major regulators of TSH production and inhibit its secretion by anterior pituitary thyrotropes (Shupnik, Ridgway & Chin, 1989). TSH is a glycoprotein consisting of an α (common to some other hormones) and a β subunit (specific to TSH). Negative TREs are found in both the α subunit gene and the TSHβ subunit gene for both rats and humans (Williams & Brent, 1995). Although TRs bind T3 with greater affinity than T4, and exogenous T3 can inhibit TSH secretion, serum TSH levels are more negatively correlated with serum T4 than serum T3 levels (see Chopra, 1996). The relative ability of thyroid hormones to inhibit TSH release will be a combination of their uptake, intracellular metabolism and relative binding to the nuclear TRs. Everts *et al*. (1995) compared the relative uptake, metabolism and inhibition of TSH release (by cultured rat anterior pituitary cells) of thyroid hormones and their deaminated products, Tetrac and Triac. Relative to the free concentration, cellular uptake rates were in the following order; Tetrac > T3 > T4 > rT3. Whereas T3 was not metabolised, Tetrac, T4 and rT3 were all partly metabolised by the cultured anterior pituitary cells. The order of potency for reducing TSH release was Triac > T3 > Tetrac > T4 but it seems that for both Tetrac and T4 it is likely that their effect is indirect and possibly due to their respective metabolites following intracellular deiodination. Although nuclear receptors show negligible affinity for rT3 and T2, these two iodothyronines exhibited *in vitro* inhibition of TSH release. These effects were observed at concentrations above normal and would thus seem to have little *in vivo* relevance. They do, however, raise mechanistic questions about possible additional non-nuclear receptor influences on TSH secretion.

Once secreted into the circulation most thyroid hormone molecules are bound to plasma proteins. TBG has two possible positive TREs, one in intron 0 and the other in the 3′ region of the gene (Akbari *et al*., 1993). In monkey hepatocarcinoma cell culture, increasing T4 concentration in the medium from 0.01 pM to 10 pM increased TBG appearance whilst further increases of T4 concentration to 10 nM resulted in a decrease in TBG appearance in the medium (Gershengorn *et al*., 1976). In humans, treatment of thyrotoxicosis resulted in a 24% increase in TBG concentrations although there was considerable overlap in TBG levels measured in thyrotoxic and hypothyroid patients (Burr *et al*., 1979). The role of these putative TBG TREs is not clear. Another T4 binding protein, TTR, is unresponsive to thyroid status in that the levels of TTR mRNA in liver and choroid plexus were unchanged during both hypothyroidism and hyperthyroidism in the rat (Blay *et al*., 1993). Similarly, plasma albumin concentrations are not known to be changed by alterations in thyroid status in the adult. During hypothyroidism in humans there are changes in the binding of both T4 and T3 to plasma lipoproteins,

but no changes in the distribution of rT3 binding to the various lipoprotein classes (Benvenga & Robbins, 1996).

Changes in the thyroid hormone pool in intestinal contents will diminish the decrease in plasma thyroid hormone levels following thyroidectomy (DiStefano *et al.*, 1993). In euthyroid rats, the amount of T3 in the intestinal contents is 20 times the plasma pool size and this T3 is exchangeable with the circulation. For example, T4 and T3 levels in the hepatic portal vein plasma are, respectively, 15% and 30% greater than that in systemic plasma in euthyroid rats. In euthyroid rats, 34% of infused T3 is excreted in the faeces; however, following thyroidectomy this decreases to 20% of infused T3, and the hepatic portal plasma concentrations of T4 and T3 are 95% and 63% greater than the respective concentrations in the systemic plasma. Whereas the plasma T3 concentration decreases by 64% in thyroidectomized rats, T3 concentration in the intestinal contents decreases by 85%. (DiStefano *et al.*, 1993).

To my knowledge, it is not known if any of the cellular uptake mechanisms for thyroid hormones are influenced by thyroid status.

Intracellular deiodinases are also influenced by thyroid status. D1 deiodinase activity in rat liver and kidney is increased in hyperthyroidism and decreased in hypothyroidism (Kaplan & Utiger, 1978) and this appears to be a TR-mediated effect in that two functional positive TREs have been identified upstream of the human D1 gene (Toyoda *et al.*, 1995). However although mouse D1 gene expression is increased by thyroid hormone the upstream region of the mouse gene does not seem to contain a TRE and thus its control may be different to that of the human D1 gene (Maia *et al.*, 1995).

The activity of the D2 deiodinase is inversely related to thyroid status (Kaplan, 1986). This is the opposite of the influence of thyroid status on D1 and, unlike D1, this has been shown to be a non-nuclear-mediated action of the thyroid hormones. In the absence of thyroid hormones, the D2 activity of astrocytes in cell culture was 10-15-fold greater than when they were grown in the presence of serum and it was demonstrated that this increase in D2 activity was due to decreased removal of the enzyme with no change in its rate of synthesis (Leonard *et al.*, 1990). T4 and rT3 had similar potency whilst T3 was 100-fold less effective, which is similar to *in vivo* findings (Silva & Leonard, 1985). The thyroid-hormone-sensitive degradation/inactivation of the membrane-bound D2 is an energy-requiring process requiring an intact actin cytoskeleton (Leonard *et al.*, 1990) and involves specific interactions between the D2 enzyme, F-actin and T4 molecules (Farwell & Leonard, 1992). Investigation of the degradation and recycling of a D2 subunit in astrocytes suggests that T4 influences fundamental processes involved with the turnover of integral membrane proteins (Farwell *et al.*, 1996). Decreases in D2 mRNA levels in the cerebral cortex of hypothyroid rats have been recorded following injections of both T4 and T3 (Burmeister, Pachucki & St. Germain, 1997). The inhibitory effects of T4 on the D2 enzyme are rapid, being evident within 15 min of addition of hormone to cultured GH3 cells (St Germain, 1985). T4 is more potent than T3 in influencing D2 mRNA levels. This thyroid hormone effect is not mediated by nuclear TRs.

Thyroid hormones influence the D3 deiodinase in a similar manner to their effect on the D1 deiodinase. Hypothyroidism results in a decrease in skin D3 activity in the rat (Huang *et al.*, 1985) whilst thyroid hormones induce D3 activity in cultured astroglial cells (Esfandiari *et al.*, 1992). However, D3 activity in the placenta is unaffected by thyroid status in the rat (Emerson *et al.*, 1988). Expression of the mRNA for D3 in cultured cell lines from the amphibian *Xenopus laevis* is increased in the presence of T3 (St. Germain *et al.*, 1994). Although to my knowledge no TRE has been described associated with the D3 gene, this effect of thyroid hormones is likely to be mediated by thyroid nuclear receptors.

Although levels of mRNA for TRβ-1 in rat heart, kidney, liver and brain are unaffected, in the pituitary supraphysiological doses of T3 increase TRβ-1 mRNA levels (Hodin, Lazar & Chin, 1990). Similarly, in the rat around birth there is a dramatic increase in TRβ-1 mRNA levels associated with an increase in T3 concentration (Strait *et al.*, 1990) and in *Xenopus laevis* during metamorphosis there is an up-regulation of TRβ-1 concentration mediated by increasing T3 levels (Kanamori & Brown, 1992). That these tissue-specific and developmental-time-specific increases in TRβ-1 mRNA levels are TR-mediated effects is supported by the finding of two TREs in the promoter region of the human TRβ gene (Suzuki *et al.*, 1994). The fact that a supra-physiological T3 dose resulted in an increase in TRβ-1 mRNA levels in the pituitary in the rat is, however, complicated by the observation that it also resulted in a simultaneous decrease in levels of TRβ-2 mRNA (Hodin *et al.*, 1990) which is transcribed from the same gene. In the anterior pituitary of the rat, the total nuclear TR binding capacity and isoform distribution is relatively unaffected by

changes in thyroid status, either hypothyroidism or hyperthyroidism (Ercan-Fang, Schwartz & Oppenheimer, 1996).

Thyroid hormones have effects at almost every level of the thyroid hormone axis and together these effects appear to function as a multi-level, hierarchical system that, in the adult, acts homeostatically to maintain a relatively constant cellular level of T3 in some tissues (notably the brain). All but two of these effects (namely the effect on D2 and the enterohepatic changes) appear to be mediated by nuclear thyroid receptors. The effects on TRH and TSH secretion are part of the classic negative feedback system that acts to maintain a relatively constant plasma T4 concentration. The enterohepatic effects of hypothyroidism will also act to dampen fluctuations in plasma T4 concentration. The high-affinity-binding plasma proteins in different vertebrates can be regarded as mechanisms maintaining a relatively constant level of plasma free T4 which in turn will facilitate an even distribution of T4 to the tissues both with respect to time and body location. The secretion of TTR by the choroid plexus into the cerebrospinal fluid of higher vertebrates probably has a similar function. The effect of thyroid status on the three deiodinases, and their respective tissue distribution can be interpreted as a system to maintain a constant level of cellular T3 in select tissues, especially the brain. During hypothyroidism, T4 consumption will be minimized in those tissues which have D1 (e.g. liver and kidney) whilst intracellular T3 content will be maintained in those tissues that have the D2 enzyme, while the decreased D3 activity will minimize T3 removal. This means that especially within the brain there is strong homeostasis of cellular T3 levels. For example, brain T3 levels are at euthyroid levels when hypothyroid rats are given T4 at only 10% of the normal replacement dose (Silva & Leonard, 1985). In hypothyroidism, the rat brain makes almost all of its own T3 from intracerebral T4, whereas during hyperthyroidism, it derives most of its T3 from the circulation (Dratman *et al.*, 1983). A similar response has been recorded in the rat fetus during hypothyroidism (Ruiz de Ona *et al.*, 1988; Morreale de Escobar *et al.*, 1989). Whether such homeostasis is present in other tissues is not known but one study suggests that it may also occur in brown adipose tissue (van Doorn, Roelfsema & van der Heide, 1986). This mechanism, unknown at the time, is likely to be responsible for the early reports of the brain being unresponsive to changes in systemic thyroid status.

It appears that, extracellularly, the 'regulated' variable is free T4, whilst intracellularly it is T3. There is a hierarchy of tissues with respect to the maintenance of intracellular T3 levels. This hierarchy is manifest in the results of Escobar-Morreale *et al.* (1996) where some tissues (notably cerebellum and cerebral cortex) maintained a relatively constant tissue T3 concentration whilst in others it was variable (e.g. muscle, adrenal, ovary and heart).

Currently, there is increasing interest in 3,5-T2, a deiodination product of T3. The 'homeostatic-like' influence of thyroid status on the deiodinase enzymes means that plasma 3,5-T2 concentration will also vary less than plasma T3 levels during changes in thyroid status. For example, Faber *et al.* (1982) found that although serum T3 levels changed by +82% and −71% (relative to euthyroid values) in hyperthyroidism and hypothyroidism, respectively, serum 3,5-T2 concentration changed by only +54% and −27 %, respectively (this last change was not statistically significant). Recently, it has been shown that 3,5-T2 can inhibit TSH secretion from the anterior pituitary cells of rats both *in vivo* and *in vitro* (Horst *et al.*, 1995) and that it also stimulates pituitary D1 activity in rats (Baur *et al.*, 1997). Although nuclear receptors show negligible affinity for T2, its *in vivo* potency is not greatly dissimilar from that of T3 and this finding raises intriguing possibilities concerning the mode of action of thyroid hormones. Measurement of normal intracellular levels of 3,5-T2 are necessary before we can be sure of the physiological relevance of these findings.

(4) Effects on metabolism and thermogenesis

The stimulatory effects of thyroid hormones on metabolic activity (and thus heat production) have been known for over a century (Magnus-Levy, 1895). Indeed, before the advent of modern hormone assays, the measurement of basal metabolic rate (BMR) was a diagnostic test for the thyroid status of individuals (e.g. Dubois, 1936).

The finding that the allometry of T4 and T3 utilization by mammals has the same exponents as their BMR relationship (Tomasi, 1991) means that there is a stoichiometric relationship in all mammals, whether mice or mammoths, whereby consumption of one mole of oxygen is associated with the consumption of approximately 6.3 pmol of T4 and approximately 0.63 pmol of T3.

Thyroidectomy is capable of decreasing BMR by up to 40% in humans (Dubois, 1936) and has a

similar effect in other mammals and in birds. Although it was originally believed that the thyroid was not capable of stimulating metabolism in ectothermic vertebrates (and this statement is repeated in some recent reviews, e.g. Freake & Oppenheimer, 1995; Oppenheimer, Schwartz & Strait, 1995), it was shown some time ago that whether thyroid hormones stimulate the metabolism of reptiles and amphibians depended on their body temperature (Maher, 1961, 1964, 1967). It has since been confirmed that thyroid status affects metabolism in ectotherms (e.g. John-Alder, 1983) and that this effect is body-temperature-sensitive (e.g. Hulbert & Williams, 1988). Joos & John-Alder (1990) have shown that T4 supplementation stimulated standard metabolic rate (SMR, analogous to BMR) by 20 % in laboratory-captive lizards and by 59 % in the field-active individuals. Although night-time ambient temperatures were not different between the laboratory and field, day-time ambient temperatures were greater in the field than the laboratory. An interesting finding from this study is that T4 supplementation resulted in a decrease in plasma T3 levels and SMR was correlated with plasma total T4 concentration but not T3 levels. For a review of thyroid influence on ectotherm metabolic activity the reader is referred to Gupta & Thapliyal (1991).

Metabolic activity is quite sensitive to plasma thyroid hormone concentration. In a recent study of patients receiving chronic thyroid hormone replacement, small changes in their T4 dose significantly affected their resting energy expenditure. Whilst plasma TSH concentration was most sensitive to small changes in T4 dose, resting energy expenditure was the next most sensitive parameter measured and thyroid-sensitive blood variables such as levels of plasma sex hormone binding globulin, triglycerides, lipoprotein cholesterol levels and angiotensin converting enzyme showed no overall change. The low T4 daily dose used in this study averaged 98 μg T4 while the highest dose used was an average 141 μg T4 per day (respectively, equivalent to approximately 0.25 μg and 0.35 μg 100 g body mass per day). This small change in daily T4 dose raised plasma free T4 levels from 18 pM to 23 pM and resulted in an average 6 % increase in resting energy expenditure (Al-Adsani, Hoffer & Silva, 1997).

In euthyroid humans, BMR was found to be positively correlated with total T3 concentration (Stenlof *et al.*, 1993). Another study found that daily energy expenditure of healthy humans was correlated with plasma free T3 levels (Toubro *et al.*, 1996). A comparison of post-obese woman with matched (never obese) controls demonstrated that the previously obese subjects had a low BMR and low plasma free T3 levels (Astrup *et al.*, 1996). In that study, the lower plasma free T3 levels could statistically explain the low BMR of the obesity-prone individuals but whether there was a causal connection could not be established. These correlations between metabolism and T3 concentration may be due to a low metabolic activity being associated with low deiodination activity and thus a low production rate of T3.

Thyroid hormones can also affect other measures of metabolic activity. For example, thyroidectomy decreases work rate and work efficiency in goats (Kaciuba-Uscilko *et al.*, 1987) and decreases aerobic capacity (Vo_{2max}) and locomotor endurance in lizards (John-Alder, 1984*a*).

That the stimulation of metabolic activity by thyroid hormones is largely a cellular event is evident from early studies which showed that tissue slices isolated from rats of different thyroid status had different *in vitro* respiration rates (Barker & Klitgaard, 1952). Most tissues appear to be thyroid hormone sensitive. The conclusion from early studies that brain was refractory to thyroid stimulation of metabolism should be re-examined in view of our current understanding of the relative constancy of brain T3 levels in different thyroid states. Much biochemical work has been carried out on isolated enzymes and sub-cellular preparations (e.g. mitochondria) from animals of varied thyroid status in order to identify the cellular processes important in this thyroid-stimulated metabolic activity. However, as with many *in vitro* measurements, the physiological relevance of many of these findings has been difficult to determine because of the problem of extrapolation to the *in vivo* situation. The development of metabolic control analysis and its associated techniques over the last quarter of a century has allowed a more quantitative analysis of the importance of various cellular processes during thyroid stimulation of metabolism (see Harper & Brand, 1995). For a review of the use of top-down regulation analysis in understanding energy metabolism the reader is referred to Brand (1997).

Basal metabolic rate varies dramatically between vertebrates. Endotherms (mammals and birds) have BMRs that are 5–10 times those of ectothermic vertebrates of the same body size (Hulbert, 1980*b*) and BMR varies allometrically with body mass in a very predictable manner such that per gram body mass, a mouse has a BMR that is approximately 20 times that of a horse (Kleiber, 1961). Part of these

differences are due to variations in the relative size of metabolically active organs but a substantial part of this BMR variation is related to basic cellular differences. A surprising finding has been that, although there is considerable quantitative difference in cellular metabolic activity, the relative composition of cellular metabolic rate is relatively constant for a particular tissue between different vertebrates. This has led to the suggestion of a cellular 'pacemaker' for metabolic rate. The finding that membrane polyunsaturation is positively correlated with metabolic activity both in phylogenetic comparisons (Brand *et al.*, 1991; Brookes *et al.*, 1998) and body size comparisons (Couture & Hulbert, 1995*a*, *b*; Porter, Hulbert & Brand, 1996) has led to the suggestion that membranes are such 'pacemakers' of metabolism (Hulbert & Else, 1999, 2000).

Although the precise quantitative contribution varies between cell types, the major energy-consuming processes of resting cells are: (i) maintenance of the mitochondrial membrane H^+ gradient, (ii) maintenance of the plasma membrane Na^+ gradient, (iii) maintenance of low cytosolic $[Ca^{2+}]$, (iv) synthesis of macromolecules (DNA, RNA, and protein), and in some cells (e.g. liver) processes such as ureagenesis and gluconeogenesis (for a recent review, see Rolfe & Brown, 1997). In active muscle cells, considerable energy consumption is associated with contractile activity. All but the first of these activities are mediated by ATP consumption.

In active cells, the mitochondrial proton gradient is used to produce ATP for the ATP-consuming processes. In resting cells, substrate oxidation continues but in this case, when ATP consumption is minimal, proton pumping counteracts the leak of protons across the mitochondrial inner membrane. In this context, the mitochondrial proton leak is analogous to the 'governor' of a steam engine, in that, just as the governor of a steam engine prevents explosion of its boiler, proton leak (which is non-linearly related to the mitochondrial membrane potential) acts to counteract the continual build-up of the trans-membrane proton gradient and thus the mitochondrial membrane potential. For a discussion of mitochondrial proton leak, the reader is referred to Brand *et al.* (1994) and Rolfe & Brand (1997).

The maintenance of transmembrane ion gradients is important in that many other cellular processes are linked to such gradients. They can be thought of as a form of short-term energy storage. Studies on rat thymocytes show there is a hierarchy of cellular ATP-consuming processes to changes in energy supply, with the maintenance of ion gradients being the least sensitive and protein synthesis being the most sensitive to changes in energy supply (Buttgereit & Brand, 1995). The maintenance of ion gradients represents a major energy cost of the non-equilibrium condition we know as life.

In hepatocytes isolated from rats, hypothyroidism and hyperthyroidism resulted in, respectively, a decreased and increased oxygen consumption relative to littermate euthyroid controls (Harper & Brand, 1993). Top-down elasticity analysis showed that in the hypothyroid-euthyroid comparison, half the increase in hepatocyte oxygen consumption was due to an increased mitochondrial proton leak and half was due to an increase in non-mitochondrial oxygen consumption with no net change in ATP turnover. In the hyperthyroid-euthyroid comparison, just under half the increase was due to a greater mitochondrial proton leak, whilst just over half was due to an increase in ATP turnover and there was no significant change in the non-mitochondrial oxygen consumption (Harper & Brand, 1993). Even when rat hepatocytes are performing considerable ureagenesis and gluconeogenesis, thyroid hormones stimulate both coupled respiration (that associated with ATP production) and non-coupled respiration (i.e. either mitochondrial proton-leak-associated respiration or non-mitochondrial oxygen consumption, or both) (Gregory & Berry, 1992).

Thyroid status influences proton leak in isolated rat liver mitochondria with the hypothyroid-hyperthyroid transition resulting in a sevenfold increase in the proton permeability of mitochondria (Hafner *et al.*, 1988). Related to this effect, hyperthyroidism results in a mitochondrial membrane potential that is approximately 30 mV lower than euthyroid controls when measured in intact resting rat hepatocytes (Gregory & Berry, 1991). Other workers have shown that in intact rat hepatocytes, the mitochondrial membrane potential is greatest in hepatocytes from hypothyroid rats and least in those from hyperthyroid rats; however, respiratory rate showed the opposite trend being least in hypothyroid and greatest in hepatocytes prepared from hyperthyroid rats (Bobyleva *et al.*, 1998). As these authors suggest this indicates a physiological effect (rather than a pathological consequence) of thyroid hormones decreasing mitochondrial energy coupling.

Mitochondrial proton leak is found in all the major oxygen-consuming tissues of the rat (Rolfe, Hulbert & Brand, 1994), and in the liver mitochondrial proton leakiness has been shown to be correlated with the relative polyunsaturation of the mitochondrial membrane (Porter *et al.*, 1996;

Brookes *et al.*, 1998). An examination of the mechanistic basis for the increased mitochondrial proton permeability induced by thyroid hormones suggested that two factors were responsible. These were: (i) changes in the relative amount of mitochondrial inner membrane, and (ii) changes in the intrinsic permeability of the phospholipid bilayer from the mitochondrial inner membrane (Brand *et al.*, 1992). Such permeability changes are not restricted to proton permeability in that hypothyroidism has also been shown to result in a decreased inner membrane permeability to other cations in rat liver mitochondria (Hafner, Leake & Brand, 1989).

Metabolic control analysis of liver mitochondria has shown that control of mitochondrial respiration is distributed among a number of steps and that effective stimulation by thyroid hormones can only occur if several enzymes are activated simultaneously (Verhoeven *et al.*, 1985). In that study, thyroid hormone treatment resulted in an increase in the flux control coefficient of the adenine nucleotide translocator, the dicarboxylate carrier and cytochrome oxidase. Sterling & Brenner (1995) have convincingly shown that low doses of thyroid hormones stimulate adenine nucleotide translocator activity in rat liver mitochondria.

Many studies have reported an effect of thyroid status on the activity of a wide variety of mitochondrial enzymes from a variety of tissues, with an increase in thyroid status generally associated with greater enzyme activity. For a review on thyroid hormone action on mitochondria, the reader is referred to Soboll (1993). Thyroid hormones both result in more mitochondrial membrane and an altered membrane lipid composition (e.g. see Tables 2 and 3). Changes in mitochondrial membrane lipid composition are associated with changes in the Arrhenius kinetics of mitochondrial enzyme activity that is also associated with changes in temperature-induced phase transitions of the mitochondrial membrane (Hulbert, Augee & Raison, 1976; Parmar *et al.*, 1995).

The role of thyroid-induced changes in membrane lipid composition is also of interest in relation to changes in the activity of some mitochondrial enzymes. The transport of pyruvate and tricarboxylates is stimulated by thyroid hormones in rat liver mitochondria in that the maximal rate (V_{max}) of the process is increased, yet inhibitor studies suggest that there is no increase in the number of any of these membrane transporters (Paradies & Ruggiero, 1990*a*, *b*; Paradies, Ruggiero & Dinoi, 1991). Similarly, in rat heart mitochondria thyroid hormones stimulate pyruvate translocation and cytochrome oxidase activity, without equivalent changes in the amount of the specific mitochondrial enzyme (Paradies & Ruggiero, 1989; Paradies *et al.*, 1993, 1994). These studies also report thyroid-induced changes in mitochondrial membrane lipids and suggest that the membrane lipid changes influenced the molecular activity of these mitochondrial membrane proteins. In a similar vein, Beleznai *et al.* (1989) demonstrated that T4 dramatically influenced the physical properties of the lipid microenvironment of the mitochondrial enzyme, L-glycerol-3-phosphate dehydrogenase.

It seems likely that thyroid effects on mitochondrial function can best be described as the consequence of thyroid-hormone-induced changes in membrane lipid composition, especially changes in fatty acyl composition. A thyroid-hormone-induced increase in the relative polyunsaturation of mitochondrial membranes (e.g. decreased 18:2 combined with increased 20:4) is proposed to stimulate the molecular activity of proteins associated with the membranes (see Hulbert & Else, 1999). No mitochondrial enzymes are among those genes reported to have a TRE (see Williams & Brent, 1995).

Thyroid hormone stimulation of cellular ATP turnover requires an increase in ATP consumption as well as ATP production. As noted above, the maintenance of the Na^+ gradient across the plasma membrane of cells is a major cellular energy consumer, the importance of which varies between tissues (for review see Clausen, van Hardeveld & Everts, 1991). This gradient is maintained by the ubiquitous Na^+,K^+-ATPase, also known as the Na^+ pump. Almost 30 years ago, Ismail-Beigi & Edelman (1970, 1971) proposed that a substantial portion of the enhanced oxygen consumption associated with increased thyroid status is due to thyroid hormone stimulation of the Na^+ pump. Although there was considerable discussion about the quantitative importance of this effect, there was no disagreement that Na^+ pumping was stimulated by thyroid hormones. The disagreement about the quantitative importance is largely based on methodological considerations. For example, in rat liver, ouabain inhibition of the Na^+ pump results in a 30–40% decrease in respiration (e.g. Hulbert & Else, 1981) whilst measurement of pump activity suggests that its ATP consumption is equivalent to approximately 10% of liver cell oxygen consumption (e.g. Couture & Hulbert, 1995*b*). Measurement of Na^+ pump

activity represents the cost of 'maintaining' the Na^+ gradient, whilst measurement of ouabain inhibition of respiration measures the cost of 'not maintaining' this gradient and will thus include inhibitory effects of elevated intracellular Na^+ concentrations as well as the energy consumption by the pump itself.

The initial report that thyroid hormones stimulated the ouabain-suppressible oxygen consumption of tissue slices (Ismail-Beigi & Edelman, 1970) was followed by the demonstration that the *in vitro* enzymatic activity of the Na^+ pump was also increased (Ismail-Beigi & Edelman, 1971). This effect has been reported for a wide variety of tissues (including liver, kidney, heart, skeletal muscles and adipose tissue) and several studies have shown that thyroid hormones increase pump numbers and sub-unit mRNAs (for review see Ismail-Beigi, 1993). A comparison of sub-unit mRNAs and Na^+,K^+-ATPase protein levels in a range of tissues has shown there are differential effects of the thyroid hormones both between tissues and between subunits and that these include both pre- and post-translational influences (Horowitz *et al.*, 1990).

An obvious question concerns the mode of action for this thyroid hormone effect. It is thus of interest that Haber & Loeb (1984) reported a thyroid-hormone-induced increase in K^+ efflux from rat liver slices that preceded changes in Na^+,K^+-ATPase activity. Similarly, Everts & Clausen (1988) reported increases in passive Na^+ and K^+ leaks that preceded changes in ouabain binding (a measure of Na^+ pump numbers). The finding that T3-induced increases in Na^+,K^+-ATPase activity were not preceded by changes in intracellular ion concentrations (Philipson & Edelman, 1977) may be related to the fineness of the homeostatic control of these variables by the Na^+ pump and the difficulty of measuring intracellular ion concentrations.

Measurement of thyroid-hormone-induced changes in a rat liver cell line showed a significant 40% increase in Na^+ influx 12 h, and a significant 10% increase in K^+ efflux 8 h after T3 exposure (Haber, Ismail-Beigi & Loeb, 1988). The increase in *in vivo* pump activity became statistically significant 12 h after hormone exposure and *in vitro* Na^+,K^+-ATPase activity only became statistically significant after 16 h. In addition, the increase in *in vivo* pump activity was consistently greater than the increase in *in vitro* enzyme activity (Haber *et al.*, 1988). An intriguing possibility is that the increase in passive Na^+ flux is secondary to a thyroid-hormone-induced increase in the fluxes of other molecules whose transport is sometimes linked to the Na^+ gradient (e.g. possibly Ca^{2+}, glucose, amino acids etc.). In this light, it is of interest that the uptake of deoxyglucose was significantly increased after 4 h of hormone exposure, as was lactate efflux (Haber *et al.*, 1988). Similarly, Everts & Clausen (1988) reported that T3 induced an increase in passive K^+ efflux (measured as Rb^+ efflux) from rat soleus muscle that preceded the increase in muscle Na^+,K^+-ATPase activity by 1–2 days.

This perspective is also supported by the finding that short-term administration of T3 to humans resulted in no change in Na^+ pump numbers in leucocytes but a significant increase in uptake rate of Rb^+ (a commonly used experimental analogue for K^+) and rate of Na^+ efflux. This indicates an increased molecular activity of the Na^+ pumps but no change in their number in these cells (Turaihi *et al.*, 1987). Similarly, it has been demonstrated that T3 administration to thyroidectomized rats stimulates fluid reabsorption in their kidney proximal tubules (which is related to Na^+ pump activity) but did not change the *in vitro* Na^+,K^+-ATPase activity, suggesting no change in Na^+ pump concentration. Other experiments indicated that this thyroid hormone effect on *in vivo* Na^+ pump activity resulted from an increase in the K^+ permeability of the proximal tubular cell membranes (Capasso *et al.*, 1985).

One of the main pathways of Na^+ entry into cells is *via* the (amiloride-sensitive) Na^+/H^+ exchanger in the plasma membrane. Thyroid status has been shown to stimulate Na^+/H^+ exchange in renal brush border membranes isolated from the rat although it is not known if this is due to an increase in the number of exchangers or an increase in their molecular activity (Kinsella & Sacktor, 1985).

Whether such changes are related to changes in the acyl composition of the plasma membrane is unknown but it is well documented that the activity of Na/K ATPase as well as other aspects of transmembrane ion movements are influenced by membrane lipids (see Hoch, 1988; Hulbert & Else, 2000). A survey of the upstream region of the human α sub-unit gene failed to find a TREsequence (Shull, Pugh & Lingrel, 1989) and none of the Na/K ATPase sub-units are among the genes reported to possess a TRE (Williams & Brent, 1995).

The most parsimonious explanation is that the increased nuclear expression of Na^+,K^+-ATPase units is a response to an increase in passive ion leaks. This increased leak results in an increased activity of the Na^+ pumps already present in the membrane and later an increase in the number of pumps. Such

a mechanism, whereby the demand for ion transport is the primary driving force for Na^+ pump synthesis, has been suggested by Wolitzky & Fambrough (1986) and is supported by the finding that exposure of chicken muscle cells to veratridine (which increases Na^+ leak by opening Na^+ channels) results in an increase in both the activity of and the mRNA for Na^+,K^+-ATPase (Taormina & Fambrough, 1990).

Erythrocytes from hyperthyroid humans have both an altered membrane phospholipid profile and show an increased Na^+ and Ca^{2+} influx compared to euthyroid controls (De Riva, Virgili & Frigato, 1996). Whilst there were no significant changes in either phospholipid profile or ion fluxes 30 days after commencement of treatment (plasma thyroid hormone levels were reduced but still in the hyperthyroid range), 300 days after treatment commenced both phospholipid profile and ion fluxes had returned to euthyroid levels. Because mammalian erythrocytes have no intracellular organelles it is likely that individual cells are unable to modify their membrane composition and it is necessary for population turnover to occur for changes to be manifest (De Riva *et al*., 1996).

The Ca^{2+} gradient across the plasma membrane of resting cells is the largest known for any ion (being approximately $\times 10^4$) and is maintained by a Ca^{2+}-ATPase and a Ca^{2+}/Na^+ exchanger, both in the plasma membrane, as well as another Ca^{2+}-ATPase in the endoplasmic reticulum and a Ca^{2+} carrier in the mitochondrial membrane. T3 rapidly stimulates Ca^{2+} uptake by the perfused rat liver (Hummerich & Soboll, 1989). Ca^{2+} cycling has been most studied in muscle (for review see Clausen *et al*., 1991). Thyroid hormones have a greater effect on slow muscle than fast muscle and have been shown to result in an increased amount of sarcoplasmic reticulum, a greater density of Na^+ pumps and a decreased energetic efficiency of Ca^{2+} pumping. The relative contribution to the BMR depends on the degree of futile Ca^{2+} cycling that occurs in the resting state. It is estimated that approximately 30 % of the change in muscle metabolic rate from the hypothyroid to euthyroid state is due to Ca^{2+} cycling changes in the sarcoplasmic reticulum (Clausen *et al*., 1991) and that Ca^{2+}-cycling is responsible for approximately 6 % of the resting metabolism of skeletal muscle, 18–36 % in contracting cardiac muscle and approximately 2 % in liver. It is estimated that this Ca^{2+}-cycling in skeletal muscle, heart and liver accounts for 3–4 % of the BMR of a rat and possibly 4–6 % in humans (Rolfe & Brown, 1997).

The very high Ca^{2+} gradient across the plasma membrane and its low concentration in the cytosol makes Ca^{2+} entry an ideal information transfer process across the plasma membrane. To my knowledge, stimulation of Ca^{2+} entry represents the fastest known response to physiological concentrations of thyroid hormones, being evident within 15 s of *in vitro* T3 exposure in thymocytes (Segal & Ingbar, 1984) and heart tissue where the effect was evident at 10 pM (Segal, 1990*a*). It has been proposed that this Ca^{2+} influx is the first messenger in several thyroid hormone effects at the plasma membrane (for review see Segal, 1989*b*, 1990*b*). This thyroid-hormone-induced increase in intracellular Ca^{2+} concentration has also been measured in individual rat myocytes (Lomax *et al*., 1991). Such a rapid effect is obviously non-genomic in origin but whether it is associated with thyroid-hormone-induced changes in the physical properties of the bilayer is not known. It is a transient response with intracellular $[Ca^{2+}]$ being rapidly returned to normal levels.

The plasma membrane Ca^{2+}-ATPase has also been shown to be stimulated by *in vitro* thyroid hormone exposure in both rat erythrocytes (Galo, Unates & Farias, 1981), human erythrocytes (Davis *et al*., 1983) and rabbit myocardial vesicles (Rudinger *et al*., 1984). Erythrocytes isolated from hypothyroid and hyperthyroid humans have similar changes in Ca^{2+}-ATPase activities (Dube *et al*., 1986) demonstrating *in vivo* relevance.

Plasma membrane Ca^{2+}-ATPase activity is known to be affected by membrane lipids and the interaction between the regulatory calmodulin and the pump is influenced by its lipid environment (for review see Carafoli, 1991). For example, acidic phospholipids and unsaturated fatty acids are reported to mimic the stimulatory effect of calmodulin on purified erythrocyte Ca^{2+}-ATPase (Niggli, Adunyah & Carafoli, 1981). Similarly, the *in vitro* modification of membrane acyl composition of intestinal brush border vesicles has been shown to influence Ca^{2+} transport (Kreutter, Lafreniere & Rasmussen, 1984).

In human erythrocyte membranes, long chain fatty acids have been shown to modulate Ca^{2+}-ATPase activity (Davis *et al*., 1987) and retinoic acid has been shown to be a modulator of the thyroid hormone activation of this enzyme (Smith *et al*., 1989). The plasma membrane Ca^{2+}-ATPase has been most studied in mammalian erythrocytes and it is obvious that these thyroid hormone effects can not be mediated *via* the nucleus in such non-nucleated cells. They appear likely to be due to a thyroid

influence on the membrane and there is no apparent reason why such an effect should not also be manifest in nucleated cells. This influence of thyroid hormones has been reviewed by Davis, Davis & Lawrence (1989) and these authors have suggested that the mechanism of this thyroid hormone effect may involve membrane lipids. The relative potency of various iodothyronines is also different to that for nuclear-receptor-mediated effects in that L-T4 is the most potent iodothyronine, with L-T3 and 3,5-L-T2 having approximately 75% the potency of L-T4, and rT3, D-T4 and D-T3 having no stimulatory effect on the activity of human erythrocyte Ca^{2+}-ATPase.

The study on rat erythrocytes is interesting in that these researchers have demonstrated that diet-induced changes in membrane fatty acid composition influence both basal Ca^{2+}-ATPase activity and the response to both T3 and T4 (Galo *et al.*, 1981).

Thyroid hormone stimulation of metabolism must also result in increased substrate utilization. In the 1960s, *in vitro* thyroid hormone exposure was shown to stimulate amino acid uptake in chick embryo bone (Adamson & Ingbar, 1967) and similar effects were observed in rat thymocytes (Goldfine *et al.*, 1976). T3 was also shown to stimulate deoxyglucose uptake in chick embryo heart cells (Segal, Schwartz & Gordon, 1977) and in rat thymocytes, both *in vitro* (Segal & Ingbar, 1979) and *in vivo* (Segal & Ingbar, 1984). The speed of the response together with the lack of a requirement for protein synthesis demonstrated it to be a direct effect on the plasma membrane and not mediated by nuclear receptors. Since then a number of studies have shown that thyroid hormones facilitate substrate uptake across the plasma membrane of a number of cell types. For example, the uptake of deoxyglucose by cardiac and skeletal muscle and adipose tissue is stimulated *in vivo* by physiological doses of thyroid hormones (Segal, 1989*a*). It has been proposed that this effect is mediated by intracellular increases in cyclic AMP and a transient Ca^{2+} influx (see Segal, 1990).

The stimulation of glucose uptake by chick embryo heart cells is biphasic with the initial phase (up to 6 h) involving stimulation of transporters in the plasma membrane and the second phase (6–24 h) involving the nucleus and requiring protein synthesis. The production of a monoclonal antibody with the 'configuration' of T3 has been shown to stimulate the first phase of this uptake (Gordon *et al.*, 1994). This finding supports the proposal that such an effect may be mediated by T3 binding to a plasma membrane receptor.

Thyroid-hormone-stimulation of glucose uptake by a rat liver cell line (ARL 15 cells) appears to involve an increase in both transporter numbers and the molecular activity of individual GLUT 1 glucose transporters. After 6 hours of hormone exposure deoxyglucose uptake increased by 40% but GLUT 1 transporter protein levels did not change. After 48 h of T3 exposure, deoxyglucose uptake increased by 116 % whilst transporter numbers were only 58% greater (Weinstein & Haber, 1993). In the rat heart, hyperthyroidism has no effect on either GLUT 1 protein or its mRNA levels, whilst hypothyroidism results in an increase in both GLUT 1 mRNA and protein levels (Weinstein & Haber, 1992). The same study showed that, in the rat heart, GLUT 4 mRNA levels are very sensitive to, but that GLUT 4 protein levels do not change with thyroid status. In rat adipocytes, hyperthyroidism resulted in an increase in levels of GLUT 4 transporters as well as the functional activity of these transporters. The change in functional activity of the plasma membrane glucose transporters may be related to an increase in insulin receptor affinity and insulin receptor kinase activity during hypothyroidism (Matthaei *et al.*, 1995). Whilst astrocytes cultured in T3-depleted media have a decreased number of glucose transporters relative to those cultured in T3-enriched media, short exposure (10 min) of the hypothyroid cells to T3 increased the access of the transporters to the binding molecules without a change in transporter number (Roeder *et al.*, 1988). Thyroxine treatment has been shown to have only a minor influence on insulin effects on glucose kinetics in humans (Muller *et al.*, 1995).

Thyroid hormones also stimulate the uptake of amino acids and the rapid and direct stimulation of gluconeogenesis by T3 in isolated-perfused rat liver has been suggested to be due in part to this thyroid hormone effect (Muller & Seitz, 1980).

When rat hepatocytes are incubated with glucose there is an increased respiration compared to that observed in a glucose-free medium and this glucose-enhancement of respiration is related to the prior thyroid status of the rat (Gregory *et al.*, 1996). Measured rates of glucose cycling were greater in hepatocytes from euthyroid rats compared to those from hypothyroid rats, however the contribution of this enhanced hepatic glucose cycling to the calorigenic action of thyroid hormone is minimal (Gregory *et al.*, 1996).

Thyroid hormones are also known to stimulate the

synthesis of fatty acids (lipogenesis) as well as the hydrolysis of triglycerides (lipolysis). The possibility that a thyroid-hormone-stimulated lipogenesis/lipolysis 'futile' cycle is a significant contributor to the calorigenic effect has been investigated by Oppenheimer *et al.* (1991). The early loss of body fat during induced hyperthyroidism in rats suggested that this was the primary substrate for the T3-induced increase in metabolic rate as food intake only increased some days after the commencement of hyperthyroidism. The food the rats ate (normal laboratory chow) contained only 4.5% fat and the increase in lipogenesis reached a plateau 4–5 days after the commencement of hyperthyroidism. However, the metabolic cost of this increased lipogenesis accounted for 3–4% of the total T3-induced increase in metabolic rate. Thus, a futile cycle of lipogenesis/lipolysis is an insignificant contributor to the calorigenic effects of thyroid hormones. It was suggested that the function of the thyroid-induced increase in lipogenesis was simply to maintain fat stores (Oppenheimer *et al.*, 1991).

The effect of thyroid hormones on lipogenesis is due to the increased expression of genes coding for lipogenic enzymes, especially the cytosolic malic enzyme and an enigmatic protein called S_{14} that seemed to be associated with lipogenesis in an unknown way (Goodridge, 1978; Towle, Mariash & Oppenheimer, 1980; Miksicek & Towle, 1982). Recent work suggests that S_{14} protein is located primarily in the nucleus and acts in the induction of mRNAs coding for key lipogenic, glycolytic and gluconeogenic enzymes as well as for the D1 deiodinase (Brown, Maloney & Kinlaw, 1997). That the thyroid hormone effect on lipogenesis is mediated by nuclear receptors is demonstrated by the fact that the genes for these lipogenic enzymes (malic enzyme and S^{14}) have TREs (Petty *et al.*, 1990; Zilz, Murray & Towle, 1990; Liu & Towle, 1994). Indeed, the malic enzyme TRE is a common TRE in the construction of transfected systems used to examine the mechanism of action of thyroid nuclear receptors.

The induction of malic enzyme in liver is also influenced by diet fat composition. In rats, fat-free diets result in elevated activities of malic enzyme and other lipogenic enzymes and as dietary fat content (polyunsaturated soya oil) is increased lipogenic enzyme activities decrease (Carrozza *et al.*, 1979). An absence of polyunsaturates in the diet is a powerful stimulus to malic enzyme induction. In mice fed diets that differed only in fatty acid composition, polyunsaturates resulted in a significant decrease in liver malic enzyme activity both in the euthyroid and hyperthyroid state (Deshpande & Hulbert, 1995). That these diets had no effect on metabolic rate but significantly influenced body mass suggest that lipogenesis was similarly affected. Hyperthyroidism diminished the effect of dietary fat composition on liver malic enzyme activity (Deshpande & Hulbert, 1995). Interaction between dietary fat and T3 effects on malic enzyme induction has also been observed in the rat (Clarke & Hembree, 1990). The influence of dietary polyunsaturate content on malic enzyme induction suggests that energy storage may not be the only factor to be considered but that another aspect of fatty acids, their key role in the membrane bilayer, may also be important. Possibly, stimulation of lipogenesis is part of a 'make more membrane' message.

This possibility is strengthened by the intriguing findings of Castellani, Wilcox & Heimberg (1991). These authors measured lipogenesis in perfused liver from both euthyroid and hyperthyroid rats. Whilst hyperthyroidism increased both fatty acid synthesis (lipogenesis) and oxidation, the *de novo* synthesised fatty acids were preferentially incorporated into phospholipids rather than into triglycerides (by severalfold) and were poorer substrates for oxidation than were exogenous fatty acids which were preferentially incorporated into triglycerides. These findings suggest that whilst increased lipogenesis will influence triglyceride deposition the primary function of enhanced lipogenesis may to provide fatty acids for membrane synthesis. In this light, it is interesting that one of the effects of an enhanced thyroid status is an increase in the amount of cellular membranes, for example increased mitochondrial (e.g. Jakovcic *et al.*, 1978) and sarcolemmal membranes (Szymanska, Pikula & Zborowski, 1991).

In some tissues, a substantial proportion of oxygen is consumed by non-mitochondrial processes. For example, in the rat this is estimated to be 20, 14 and 3%, respectively, of the *in vivo* oxygen consumption of liver, skeletal muscle and heart and represents approximately 8% of BMR (see Rolfe & Brown, 1997). Thyroid hormones stimulate non-mitochondrial oxygen consumption of rat hepatocytes (Harper & Brand, 1993). One of the contributors to non-mitochondrial oxygen consumption will be the desaturase enzyme systems located in the endoplasmic reticulum (Pugh & Kates, 1979). It is possible that the thyroid-induced changes in membrane fatty acyl composition (see Tables 2 and 3) are responsible for part (or all) of the thyroid-induced changes in non-mitochondrial oxygen consumption *via* altered desaturase activity.

Although when considered as whole organisms, vertebrates are aerobic organisms, some individual cell types can adopt anaerobic strategies. Approximately half the heat production of lymphocytes measured *in vitro* is associated with anaerobic energy metabolism. Lymphocytes from hyperthyroid humans have an elevated heat production due to enhanced anaerobic metabolism (Valdermarsson & Monti, 1994) that in turn appears to be due to thyroid hormone stimulation of adrenoreceptor sensitivity (Valdermarsson & Monti, 1995).

As mentioned above it is often assumed that T3 is the 'active' thyroid hormone and a large amount of the research cited in this section has involved the use of T3. A decade ago, a surprising report appeared that fundamentally questioned this assumption.

Horst, Rokos & Seitz (1989) reported that 3,5-T2, at a concentration as low as 1 pM, resulted in a rapid stimulation (within 30 min) of oxygen consumption of the perfused liver from hypothyroid rats. T2 was as potent as T3 and exerted its effect more rapidly. Whereas the stimulation by T3 was largely abolished in the presence of an inhibitor of the D1 deiodinase, the 3,5-T2 stimulation of oxygen consumption was not affected. Only T3 increased liver malic enzyme activity with this enzyme being unaffected by 3,5-T2 (Horst *et al.*, 1989). The interpretation of this finding was that the calorigenic effect of the thyroid hormones was mediated by a direct and rapid effect of 3,5-T2 (derived from the intracellular deiodination of T3) on the mitochondria whilst other longer term effects (such as increased malic enzyme activity) were mediated *via* the nucleus.

Since this initial report, both 3,5-T2 and 3,3′-T2 have been shown to be equipotent in stimulating mitochondrial cytochrome oxidase activity *in vitro*; the effects are rapid (being evident within 5 min) and are restricted to these two iodothyronines (Lanni *et al.*, 1994*a*). When large doses of 3,5-T2 or T3 were injected into anaesthetized rats that had been treated with the D1 deiodinase inhibitor, propylthiouracil (which will prevent the conversion of T3 to T2), and mitochondria were isolated 1 h later, only 3,5-T2 injections increased in mitochondrial activity (O'Reilly & Murphy, 1992). In unanaesthetized rats, 3,5-T2 was also shown to stimulate lipid β-oxidation (Cimmino *et al.*, 1996). Rat liver mitochondria have been shown to bind both 3,5-T2 (Goglia *et al.*, 1994) and 3,3′-T2 (Lanni *et al.*, 1994*b*) *in vitro*. Recently, it has been demonstrated that 3,5-T2 given as a single large *in vivo* dose resulted in a stimulation of both state 4 and state 3 respiration of rat liver mitochondria; this was due to stimulation of substrate oxidation reactions and 3,5-T2 had no effect on the proton leak nor on the phosphorylating system (Lombardi *et al.*, 1998). Both 3,5-T2 and 3,3′-T2 injected into hypothyroid rats stimulated resting metabolism (Lanni *et al.*, 1996). The latter study also showed that the relative influence of these iodothyronines on cytochrome oxidase activity varied between tissues. Hypothyroid rats responded to a single injection of either 3,5-T2 or T3 with an increase in resting metabolic rate. Although metabolic rate increased to the same degree the response to 3,5-T2 was more rapid and resistant to inhibition of protein synthesis, whilst the response to T3 was slower and completely abolished by the inhibition of protein synthesis (Moreno *et al.*, 1997). Supraphysiological levels of 3,5-T2 *in vitro* stimulated the oxygen consumption of human mononuclear blood cells but glucose uptake was unaffected (Kvetny, 1992).

The study giving the most insight into the effects of this iodothyronine on mitochondrial activity has recently shown that *in vitro* 3,5-T2 stimulates cytochrome oxidase activity. Part of the normal control of mitochondrial respiratory chain activity is due to the fact that at high ATP/ADP ratios, ATP replaces ADP bound to subunit IV of the cytochrome oxidase enzyme complex and allosterically inhibits its activity. 3,5-T2 was shown to bind to subunit Va of the cytochrome oxidase complex and abolish the allosteric inhibition due to ATP binding and thus stimulating enzyme activity (Arnold, Goglia & Kadenbach, 1998). T3 has a similar but smaller effect. This effect was first apparent at 10 nM 3,5-T2 and was maximal at 1 μM, although because of the constructed *in vitro* nature of this system these concentrations may not have *in vivo* relevance.

These studies obviously raise a number of important questions. They show that: (i) T3 is not the only active iodothyronine and that at least two T2s are also capable of being active thyroid hormones, (ii) the T2s can exert effects separate from T3 and on a molar basis are approximately as equipotent as T3 in stimulating some activities, and (iii) that this stimulation does not involve the nucleus. The later studies, however, suffer from the same general problem that plagues much of the thyroid literature. The concentrations used are generally too high to conclude physiological relevance. We currently have limited knowledge of the concentrations of these diiodothyronines in humans but very little knowledge of the levels in the blood and tissues of rats.

Whilst I know of no reports for free 3,5-T2 concentrations, the free levels for 3,3′-T2, 3′,5′-T2

and T3 are, respectively, 0.44 pM, 0.77 pM and 4.8 pM (Faber *et al.*, 1984). For a 70 kg euthyroid human, the daily production of 3,5-T2 and T3 is 6.4 nmol and 48 nmol, respectively (Chopra, 1996). Tissue concentrations of 3,5-T2 and T3 in euthyroid human brains are reported to be approximately 0.1 pmol g^{-1} and approximately 1.5 pmol g^{-1} respectively (Pinna *et al.*, 1997). Whilst the levels in adult euthyroid rats are not known, tissue 3,5-T2 and T3 content of liver from 16-day-old rat fetuses is reported to be approximately 0.4 pmol g^{-1} and approximately 3.5 pmol g^{-1}, respectively (Porterfield & Hendrich, 1992). Thus, all reports suggest that the normal *in vivo* levels of 3,5-T2 are less than those of T3 and suggest that the doses used to date are an order of magnitude greater than those required to demonstrate physiological relevance. Future studies ideally need to include the use of and confirmatory measurement of the relevant *in vivo* concentrations.

These studies may, however, provide an explanation for the conflicting findings of a direct effect of T3 on isolated mitochondria, in that if mitochondrial preparations have undetected microsomal contamination it is possible that T3 added *in vitro* could be converted to 3,5-T2 by deiodination and exert stimulatory effects on mitochondrial respiration.

That thyroid hormones can have rapid *in vivo* effects on metabolic enzymes is illustrated by a series of reports on liver and muscle from a diverse collection of non-terrestrial vertebrates, including a cyclostome (Leary *et al.*, 1997), holostean and teleost fish (Ballantyne *et al.*, 1992) and an elasmobranch (Battersby, McFarlane & Ballantyne, 1996). In all of these studies, tissues were isolated 3 h after *in vivo* injection of T3 and although T3 doses were generally in the range 1–15 μg per 100 g, in some studies effects were observed after *in vivo* T3 doses as low as 0.001 μg per 100 g. These researchers have shown a stimulation of pyruvate-fuelled state 3 mitochondrial respiration following a 5 min *in vitro* incubation with 0.3 nM T3 or 3,5-T2 (Leary, Barton & Ballantyne, 1996).

Although the effect of the thyroid hormones on basal metabolism has been known for over a century there is still no general agreement on how this effect is initiated or mediated. Part of the reason for this is that, until recently, we have not had much quantitative understanding of the processes that constitute basal metabolism. It is obvious from the discussion above that the stimulation of oxygen consumption of the whole organism is due to the stimulation of many individual processes in many different tissues. The vast majority of these processes are membrane-associated. Many involve an increase in the passage (both active and passive) of ions or substrates across membranes, and many involve an increase in the molecular activity of particular membrane-associated processes. Some are rapidly induced *in vitro* whilst some take time to become manifest. Some involve an increase in protein synthesis whilst others do not.

The evidence that these effects are initiated by thyroid nuclear receptors is scarce. The only process that has definitively been shown to be thyroid nuclear receptor initiated is the stimulus of lipogenesis and this effect is not quantitatively important. Indeed, a new interpretation of this thyroid hormone stimulation of lipogenesis is that it may be part of a 'make more membrane' message. The fact that membrane lipid composition is altered by thyroid status, and that these changes have been observed for virtually all sub-cellular membranes and in a wide variety of tissues, when coupled with the finding that membrane composition may be a 'pacemaker' in determining the BMR of different vertebrates (see Hulbert & Else, 1999, 2000) suggests that thyroid-induced changes in the composition of membrane bilayers are a significant mechanism in thyroid hormone stimulation of metabolic activity.

The proposal that thyroid hormones directly influence bilayer physical properties and that consequent changes in bilayer acyl composition are primarily responsible for mediating the calorigenic effect of thyroid hormones is compatible with many observations. It is compatible with the time lag observed before there is an increase in organismal oxygen consumption. Since bilayer acyl changes are mediated by various enzymes (especially those involved in deacylation/reacylation of phospholipids as well as phosholipid synthesis) it is compatible with the effect of inhibitors of protein synthesis. It is also compatible with the many studies that demonstrate that membrane acyl composition influences the activity of various membrane proteins, as well as being compatible with the quantitative importance of the maintenance of transmembrane gradients in cellular energy consumption. Whilst the finding that there is an increase in mRNA for a particular protein is evidence for the involvement of the nucleus in the effect, it is not evidence that the effect is initiated in the nucleus by thyroid nuclear receptors. In view of the concept of continual degradation/synthesis of proteins, an increase in protein synthesis may be due to an increased information flow to the nucleus for the particular protein. However, not all effects can

be mediated by changes in acyl composition. For example, some are too rapid. The stimulation of increased substrate uptake at the plasma membrane appears to be receptor mediated and some effects at the mitochondrial membrane may also be due to a direct interaction with proteins in the mitochondrial membrane.

The proposal that much of the stimulation of metabolic activity by thyroid hormones is mediated by changes in membrane acyl composition is unusual in that it does not require a specific receptor and therefore is not part of the current paradigm concerning hormone action. It is instead a response of the cell to the physical properties of thyroid hormone. Although we know that thyroid hormones, (at physiological concentrations) have direct physical effects on the behaviour of membranes, our knowledge of this area is still very rudimentary.

As well as effects on BMR, thyroid hormones have other effects on thermogenesis. For example, thermoregulation is also influenced by thyroid status. The lower resting body temperature of some hypothyroid mammals compared to euthyroid controls (e.g. Kaciuba-Uscilko *et al.*, 1987) is often thought to be due to a decreased metabolism and therefore thermogenesis. However, recent work using the laboratory rat suggests that rather than being a passive consequence of decreased thermogenesis this lower body temperature is instead a regulated response (Yang & Gordon, 1997) and thus may be the result of a more direct effect on the neuronal system that determines the set point for body temperature regulation.

Exposure of endothermic vertebrates (mammals and birds) to cold generally results in an increased thermogenesis to maintain body temperature homeostatically. The initial mechanism used by all endothermic vertebrates is the controlled microtremor of antagonistic muscles, known as shivering. Some of the small eutherian mammals (notably rodents) have also evolved a specialized heat-production mechanism that is located in brown adipose tissue (BAT) and controlled by the secretion of noradrenaline from the sympathetic nervous system. Heat production is the only known function of BAT and this form of heat production is important in three situations: cold-adaptation in small eutherian mammals, heat production in eutherian neonates and during arousal from hibernation in small eutherian mammals. BAT thermogenesis is not present in monotreme, marsupial or medium to large-sized eutherian mammals (e.g. see Hulbert, 1980*a*) and is also absent in non-mammalian vertebrates. It has been studied intensively in the rat and has been shown to be mediated by an uncoupling protein, UCP1, that acts to uncouple mitochondrial respiration from ATP production by facilitating proton flux through the BAT mitochondrial inner membrane (for a review see Nicholls & Locke, 1984). The selective advantage that favoured BAT thermogenesis in small eutherian mammals is presumably the fact that, unlike the tremor associated with severe shivering, this form of heat production in the cold does not disturb the insulative air layer around the animal and also allows muscle use for normal movement in the cold. Because of their size and consequently high relative surface area, small mammals cannot use the more energy-efficient strategy of increasing body insulation in the cold. Whilst BAT is an important energetic tissue in cold-adapted rats, the extrapolation of findings in the adult rat to other adult mammals (including humans) is generally not warranted.

The role of the thyroid hormones in BAT thermogenesis has been reviewed by Silva (1995). Brown adipose tissue has a high amount of D2 deiodinase and during cold exposure deiodinase activity is activated by norepinephrine resulting in substantial T3 production in the BAT cells. The elevated T3 concentration appears to facilitate, *via* nuclear TRs, the expression of the BAT UCP1 gene. Two TREs have been described for the rat UCP1 gene (Rabelo *et al.*, 1995) and the interaction between these TREs has been examined (Rabelo *et al.*, 1996).

UCP1 is a member of the mitochondrial membrane anion carrier family, and other proteins, related to UCP1, have recently been identified. Whilst these proteins have been called UCP2 and UCP3 because of their relatedness to UCP1, there is little direct evidence that they are indeed mitochondrial uncoupling proteins. Both UCP2 and UCP3 mRNA can be induced by thyroid hormones in some tissues of rats (Gong *et al.*, 1997; Lanni *et al.*, 1997) and it has been suggested that they have a thermogenic function. Neither UCP2 nor UCP3 is present in hepatocytes and can thus not be directly responsible for the mitochondrial proton leak measured in these cells. A thermogenic role for UCP2 and UCP3 has yet to be demonstrated. They have recently been discussed by Brand *et al.* (1999).

During acute cold exposure in many endotherms there is an increase in both thyroid activity and thyroid hormone metabolism. However, because cold exposure also often involves an increase in energy intake, it is not always obvious whether it is

cold exposure or the enhanced energy intake that is the primary influence on thyroid hormone. A series of studies on young pigs has shown that elevated energy intake has a greater effect on thyroid hormone metabolism than decreases in ambient temperature (for a review see Dauncey, 1990).

(5) Effects on excitable tissues

Before the advent of modern accurate hormone assays, the Achilles tendon reflex time (ankle jerk reflex) was proposed as a diagnostic for thyroid status in humans although it was later found to be unsatisfactory (Costin, Kaplan & Ling, 1970). The Achilles tendon reflex time averaged 0.34 s in euthyroid humans and 0.53 s in hypothyroids, with the time between the tap on the tendon and the muscle action potential being 0.03–0.04 s for hypothyroid, euthyroid and thyrotoxic individuals (DeLong, 1996). This finding suggested that there was no effect of thyroid hormones on nerve conduction velocity but that there were significant thyroid influences on the processes involved in muscular contraction. Measurement of peripheral and central nerve conduction velocity in humans showed that hypothyroidism resulted in slower peripheral and central nerve conduction velocity than in euthyroids and that this difference was eliminated after T4 treatment, but there was no difference between thyrotoxic and euthyroid individuals (Abbott *et al.*, 1983). These researchers showed that the slow conduction of the hypothyroids was largely due to the lower temperatures associated with hypothyroidism. Central nerve conduction velocity was assessed by measuring the latency of visually evoked responses. This technique has also been used to examine the effects of thyroid hormones on the visual system of rats. Whilst thyroidectomy resulted in an increased latency of the response, there was no significant difference in the optic nerve conduction velocity. The component of the rat visual system most sensitive to thyroid status was located in front of the optic chiasma, probably in the retina (Takeda, Onoda & Suzuki, 1994). In view of the relative constancy of brain T3 levels, irrespective of changes in organismal thyroid status, it is understandable that conduction velocity is unaffected by thyroidectomy. Whether peripheral nerves also maintain T3 homeostasis is unknown.

T3 has a direct effect on the cell membrane of GH3 cells in culture resulting in significant increases in membrane resistance and a hyperpolarization of the membrane potential within minutes of hormone application. Concomitant with these effects, cells spontaneously firing action potentials also stopped firing within minutes of T3 application (du Pont & Israel, 1987). Other evidence of membrane effects is the finding that cerebral cortex slices from hypothyroid mice show diminished endocytosis and uptake of amino acids and hexose compared to those from euthyroid mice. The fact that T3 can increase these activities in nerve endings within 5 min of hormone exposure demonstrates that this stimulation does not involve the nuclear receptors (Iqbal, Koenig & Trout, 1984).

Both hypothyroidism and hyperthyroidism have detrimental effects on skeletal muscle. Thyroidectomy affects the neuromuscular junction in rats, resulting in a decrease in both the number of acetylcholine receptors (Kragie & Smiehorowski, 1993) and G4 isoform acetylcholinesterase activity in fast skeletal muscle (Kragie & Stock, 1994). How these changes are initiated is not known but it was suggested that they may involve changes in muscle activity and/or alterations in the signal transduction systems regulating the G4 acetylcholinesterase isoform (Kragie & Stock, 1994).

Thyroid hormones affect transmembrane fluxes of both Na^+ and Ca^+ in skeletal muscle and thus have dramatic effects on skeletal muscle function (for review see Everts, 1996). Thyroid status in rats does not affect the peak force developed during either a single twitch or tetanus but has dramatic effects on the rate of force development and relaxation time of isolated muscles; this effect is more pronounced in slow muscles than in fast muscles. In the rat soleus, such thyroid-hormone-induced changes take several days (Montgomery, 1992).

The depolarization phase of action potentials at the sarcolemma is mediated by voltage-dependent Na^+ channels. Thyroid hormones increase both the spontaneous electrical activity and the number of Na^+ channels in cultured skeletal myotubes as well as influencing the affinity of these Na^+ channels for the neurotoxin, saxitoxin (Brodie & Sampson, 1989). Thyroid hormones also increase Na^+,K^+-ATPase activity in skeletal muscle (see Clausen *et al.*, 1991). It has been suggested that the Na^+ channels: Na^+ pumps ratio is an important determinant of the contractile endurance and rate of force recovery in muscle (Harrison, Nielsen & Clausen, 1997). In rat soleus muscle, T3 treatment increases the number of Na^+ channels and this precedes the increase in the number of Na^+ pumps; changes in contractile endurance of the isolated muscle reflect the time course of changes in the leak/pump ratio. Such

changes may be important determinants of the muscular fatigue associated with hyperthyroidism (Harrison & Clausen, 1998).

The sarcolemmal action potential initiates transfer of Ca^{2+} from the sarcoplasmic reticulum into the cytosol *via* membrane-located Ca^{2+} channels. In cultured skeletal muscle cells, T3 increases the number of Ca^{2+} channels and influences their binding affinity for a specific channel antagonist (Brodie & Sampson, 1990). The Ca^{2+} released during excitation is rapidly removed from the sarcoplasm by a Ca^{2+}-ATPase located in the sarcoplasmic reticulum. The concentration of Ca^{2+}-ATPase is approximately six times higher in fast muscle fibres than slow muscle fibres and reflects their difference in speed of relaxation following contraction (see Everts, 1996). Following T3 treatment, there is an increase in the rate of force relaxation after a sustained contraction in slow muscle, correlated with an increase in the density of Ca^{2+}-ATPase in the sarcoplasmic reticulum (Dulhunty, 1990). The latter study showed that T3 effects on fast muscle fibres were small and insignificant, and furthermore that T3 treatment results in more rapid changes in factors regulating the rate of rise in tension than those affecting tension relaxation.

Three genes encode different isoforms of the sarcoplasmic reticulum Ca^{2+}-ATPase and in fast muscle fibres SERCA1 is the predominant isoform whilst in slow skeletal muscle, SERCA2a is the main isoform expressed. T3 specifically stimulates the production of SERCA1 isoform mRNA (Simonides, van der Linden & van Hardeveld, 1990, Sayen, Rohrer & Dillmann, 1992) resulting in increased Ca^{2+}-ATPase activity and favouring the transformation of slow muscle to the fast muscle phenotype. The demonstration of TREs in the promoter region of the SERCA1 gene in a transfected system demonstrates that this effect is mediated by thyroid nuclear receptors (Simonides *et al.*, 1996). The demonstration that thyroid hormones stimulate sarcoplasmic reticulum Ca^{2+}-ATPase activity *in vitro* suggests that non-genomic effects are also possible (Warnick *et al.*, 1993). Neither mode of thyroid hormone action excludes the other.

The mode of thyroid hormone action involved in the increase in skeletal muscle Na^{+} channels (Brodie & Sampson, 1989) and Ca^{2+} channels (Brodie & Sampson, 1990) is unknown. Whilst thyroid hormone effects observed during inhibition of protein synthesis preclude a nuclear-receptor-initiated mode of action, the converse finding (i.e. no effect during the inhibition of protein synthesis) does not necessarily indicate a nuclear receptor mode of action. It does imply that the nucleus is involved, but because a thyroid effect is pre-translational does not necessarily mean it is transcriptional. It may involve a pre-transcriptional site of action. For example, the increased manufacture of Na^{+} pumps, in response to an increased Na^{+} leak, is due to a pre-transcriptional increase in information flow to the nucleus that leads to increased transcription and a consequent increase in the number of pumps. In this light, it is of interest that cytosolic Ca^{2+} has been shown to be important in the control of the amount of acetylcholine receptors and acetylcholinesterase (Birnbaum, Reis & Shainberg, 1980) and voltage-sensitive Na^{+} channels (Sherman & Catterall, 1984). All three effects are responsive to thyroid status (see above). Brodie & Sampson (1989) showed that factors that influence intracellular Ca^{2+} levels (e.g. external Ca^{2+} concentration and channel blockers) modified the thyroid hormone effect on the number of Na^{+} channels. It is possible that other thyroid hormone effects are mediated by such pre-transcriptional changes in information flow. Whether the thyroid-hormone-induced changes in skeletal muscle sarcolemmal lipid composition and fluidity (e.g. Pilarska *et al.*, 1991) will affect such information flow is not currently known.

Exposure of primary cultures of vascular smooth muscle cells to T3 results in cellular relaxation within 10 min of exposure (Ojamaa, Klemperer & Klein, 1996). The speed of the response suggests that this is non-genomic. A decrease in vascular resistance, which is compatible with this *in vitro* effect, is part of the enhanced cardiovascular hemodynamics associated with thyroid hormones *in vivo*.

The association between the thyroid gland and the heart was recognized over 200 years ago (Caleb, 1785, cited by Dillman, 1990). Indeed, cardiac hypertrophy is often used as an indication of hyperthyroidism. The *in vivo* effects of thyroid hormones on the heart are complex in that they involve a combination of direct hormone effects and indirect consequences from peripheral hemodynamic changes (Klein, 1990). The use of heterotopic cardiac transplants, in which the transplanted heart muscle receives blood from the recipient but is not hemodynamically loaded (in that it does not pump the recipients blood), has shown that cardiac hypertrophy is not a direct thyroid hormone effect. For this reason among others, many effects have been studied in isolated cardiac myocytes.

As well as enlargement of the heart, changes in

thyroid status produce alterations in the electrical activity of the heart. Tachycardia (as well as arrhythmia) is a common sign of hyperthyroidism, just as bradycardia is often observed during hypothyroidism. The duration of the cardiac action potential in isolated heart papillary muscle has been shown to be inversely related to *in vivo* thyroid status in a variety of vertebrates: rats (Di Meo et al., 1997), chickens (Di Meo *et al.*, 1993), lizards (Venditti *et al.*, 1996) and frogs (Di Meo *et al.*, 1995). Resting heart rate is directly related to *in vivo* thyroid status; the use of blockers of the sympathetic and parasympathetic nervous systems has shown that changes in the autonomic nervous system, whilst present, are not the primary cause of thyroid-hormone-induced tachycardia, and that the intrinsic heart rate is also significantly increased (Di Meo *et al.*, 1994). That thyroid-hormone-induced changes in the electrical activity of the heart may be associated with membrane lipid changes is supported by the finding that the membrane antioxidant, vitamin E, attenuates such changes in the rat (Venditti, De Leo & Di Meo, 1997*b*). Both hypothyroidism and hyperthyroidism have been shown to change the phospholipid fatty acyl composition of the sarcolemma of rabbit cardiac muscle (Szymanska *et al.*, 1991).

Studies on thyroid-hormone-induced shortening of action potential duration in the rabbit heart have suggested that changes in Na^+ pump activity and intracellular Na^+ levels can explain the changes in action potential duration (Doohan, Hool & Rasmussen, 1995). Membrane channel currents have been shown to be influenced by thyroid hormones. In neonatal rat cardiac myocytes, whole-cell-voltage-clamp studies have shown that acute exposure to T3 promotes the slow inactivation of Na^+ currents within 1–5 min after hormone addition (Craelius, Green & Harris, 1990; Harris, Green & Craelius, 1991) which suggests a direct action on the cell membrane. In rabbit cardiac myocytes, patch-clamp studies have shown that acute exposure of the extracellular side of the membrane to T3 modulated the cardiac Na^+ channels by increasing their propensity to enter a gating mode characterized by 'long events' or 'bursts' (Dudley & Baumgarten, 1993). Thyroid hormones can affect the behaviour of other membrane ion channels. For example, T3 increases the ATP sensitivity of the ATP-dependent K^+ channel from rat heart (Light *et al.*, 1998). Increased thyroid status is correlated with increased intracellular Na^+ concentrations in rat ventricular myocytes that is associated with increased intracellular acidity and it has been suggested that these effects may mediate thyroid-hormone-induced effects on cardiac contractility (Wolska *et al.*, 1997).

Thyroid hormones also affect the contractile processes of cardiac muscle. In the heart, the SERCA2a isoform of Ca^{2+}-ATPase predominates and T3 increases expression of this gene (Rohrer & Dillman, 1988). Three TREs have been described in the promoter region of the SERCA2 gene in the rat (Hartong *et al.*, 1994) and it has been suggested that this effect may be specific to TRβ-1 interacting with a myocyte-specific enhancing factor (Moriscot *et al.*, 1997). Thyroid hormones appear also to have direct effects on the sarcolemmal Ca^{2+}-ATPase in that very low concentrations of both T3 and T4 result in *in vitro* stimulation of the sarcolemmal enzyme isolated from rabbits (Rudinger *et al.*, 1984). Thyroid hormones have been shown to have an acute transient effect on the contractile abilities of the isolated rat heart that is plasma membrane mediated and Ca^{2+} dependent (Segal *et al.*, 1996). Acute extranuclear actions of thyroid hormones on the heart have been reviewed by Davis & Davis (1993).

As well as influencing cardiac contraction by affecting the proteins that regulate intracellular Ca^{2+} levels, thyroid hormones also influence the contractile proteins themselves. Myosin hydrolyses ATP and converts the chemical energy released into mechanical movement. Each hexameric myosin molecule contains four light chains and two heavy chains (MHCs). Myosin isolated from heart ventricle was found to contain three isoforms that differed only in their heavy chains (Hoh, McGrath & Hale, 1978). The V_1 isoform consists of two αMHCs, the V_2 consists of a single α combined with a single βMHC, whilst the V_3 isoform has two βMHCs. The isoforms differ in their ATPase activity with the αMHC having the higher activity. The control of MHCs in the heart has been reviewed by Morkin (1993). Thyroid hormones induce the high-activity V_1 isoform (i.e. αMHC) and repress expression of the low-activity V_3 isoform (i.e. βMHC) in the ventricle of the rat and the rabbit. This effect is mediated by nuclear receptors and TREs have been described for the promoter regions of both the αMHC and βMHC genes in both the rat and the human. The time course of *in vivo* thyroid hormone effects on MHC isoform mRNA levels in the rat heart shows that small changes are evident after 12 h and complete by 72 h after hormone administration, a time course similar to that for mRNA observed for the sarcoplasmic reticulum Ca^{2+}-ATPase (Balkman, Ojamaa & Klein, 1992). Thyroid hormones also affect the expression of MHC genes in

rat skeletal muscles where the effects are highly tissue and developmental stage specific (Izumo, Nadal-Ginard & Mahdavi, 1986).

Several other factors have been shown to influence MHC isoform expression in the heart including pressure-volume overload which promotes βMHC and decreases αMHC expression. Although cardiac ventricle MHC isoforms are very responsive to *in vivo* manipulation of thyroid status in small mammals like the rat, this is not true for larger mammals. For example, in the euthyroid baboon, dog and calf αMHC was not detectable in the heart ventricles, and following 6 weeks of high daily doses of T4, αMHC was not detected in the dog ventricle, was 17% in the baboon ventricle and 40% in the calf ventricle. In the euthyroid human, βMHC is the predominant form found in the ventricular myocardium. Little is known concerning the thyroidal regulation of cardiac MHC genes in the human, but it is unlikely to be the major mechanism underlying the inotropic action of the thyroid hormones (see Morkin, 1993).

Thyroid hormones also influence adrenergic receptors in cardiac muscle. In cultured ventricular myocytes, they increase β1 receptor number *via* an increase in transcription of the β1 adrenergic receptor gene (Bahouth, 1991). Thyroid status influences the action of catecholamines on cardiac tissue with hyperthyroidism potentiating and hypothyroidism blunting catecholamine-sensitive responses.

Interestingly, the β-adrenergic blocker, propanalol, has a favourable effect on many signs and symptoms associated with thyrotoxicosis. It decreases plasma T3 levels and increases plasma rT3 concentration and this effect appears to be due to inhibition of 5′ deiodination. This inhibition of deiodination, with its consequent effects on plasma levels of T3 and rT3, is related to membrane stabilizing activity rather than β-blocking ability (Wiersinga, 1991).

The adult brain is often thought to be relatively non-responsive to thyroid status. A recent review, however, suggests that thyroid hormones influence the expression of neuropeptides and growth factors in brain areas involved in cognitive processes in the adult but the molecular mechanisms involved in these effects are unknown (Calza, Aloe & Giardino, 1997). The spontaneous behavioural activity of adult rats has been shown to be influenced by T4 and it was suggested that this effect was due to an enhanced sensitivity to noradrenaline (Emlen, Segal & Mandell, 1972). In adult rats, the regeneration of transected peripheral nerves has been shown to be enhanced by local administration of thyroid hormone (Voinesco *et al*., 1998).

(6) Effects on growth

Hypothyroidism results in pronounced growth retardation in both endothermic vertebrates, for example, humans (Snyder, 1996), rats (Evans *et al*., 1966), and possums (Buaboocha & Gemmell, 1996), as well as in ectothermic vertebrates such as lizards (Gerwien & John-Alder, 1992), turtles (Denver & Licht, 1991) and fish (Matty, 1985). In mammals, this growth retardation is the result of both reduced secretion of growth hormone (GH) from the anterior pituitary as well as impaired peripheral growth hormone action (Snyder, 1996) and non-GH-related effects. Hypothyroidism results in lowered GHmRNA and GH content in the anterior pituitary of rats, and a reduced serum GH concentration (Samuels *et al*., 1989). In hypothyroid young humans, the nocturnal secretion of GH is dramatically reduced (Chernausek & Turner, 1989).

The early cloning of the GH gene together with the availability of rat-derived pituitary tumor cell cultures (GC and GH cell lines) allowed the first description of DNA sequences responsive to thyroid receptors (Koenig *et al*., 1987). The rat growth hormone gene has a number of positive TREs (in the upstream promoter region as well as in the third intron) and also a negative TRE (see Williams & Brent, 1995). The GH response of GH3 cells to thyroid hormones appears to be complex and thyroid-receptor specific, in that deletion of TRβ-1 results in an increase in cell TRβ-2 content and also in the basal and T3-induced GH mRNA content in these cells (Ball, Ikeda & Chin, 1997). Thyroid hormone control of the human GH gene is not as well defined, in that whilst thyroid hormone increases endogenous rat GH expression, it results in a decreased expression of transfected human GH gene (Cattini *et al*., 1986). Similarly, when the promoter regions for both the human GH and bovine GH gene were transfected into rat pituitary tumor cell cultures together with a reporter gene, the promoters for rat and bovine GH were thyroid-hormone-responsive whilst that for human GH was unresponsive to T3 (Brent *et al*., 1988).

That the relationship between thyroid status and GH secretion in other vertebrates appears not to be as simple as it is in rats and cell cultures of rat pituitary tumor cells is manifest from the observations of thyroidal inhibition of growth hormone

secretion in birds (Harvey, 1990). Age-related changes in complex feedback mechanisms, as well as appropriate experimental doses of hormones, need to be considered when examining the mechanisms of thyroid hormone influence on growth in vertebrates. High concentrations of thyroid hormones inhibit growth in many tissues (Greenberg, Najjar & Blizzard, 1974).

As with some other thyroid hormone effects, 3,5,-T2 has recently been shown to increase serum GH concentration in hypothyroid rats (Moreno *et al.*, 1998). Whilst another diiodothyronine, 3,3′-T2, has no influence on serum GH, the potency of 3,5-T2 was similar to that of T3 in these experiments. As with the other reported effects of 3,5-T2, the physiological relevance of these findings depends on measurement of the plasma and tissue 3,5-T2 concentrations. These results do however raise important questions regarding the uniqueness of T3 as the only active thyroid hormone, and regarding its mode of action, in view of the low affinity of nuclear receptors for T2.

The amount of thyroid hormones required to maintain normal growth in rats is relatively small. For example, doses as low as 0.25 μg T4/day in thyroidectomised rats resulted in growth rates similar to non-thyroidectomized controls (Evans *et al.*, 1966). This dose is approximately 25–30 % of the normal daily T4 secretion rate in rats. Although this dose resulted in restoration of near normal growth rates, it did not significantly stimulate the metabolic rate of these thyroidectomised rats which remained considerably reduced compared to non-thyroidectomized controls (Evans *et al.*, 1966). This study also presented some fascinating results in that it demonstrated that daily injections of 5 mg iodide were as effective in restoring normal growth as were T4 injections. This was proposed to be evidence for extrathyroidal T4 formation. We have confirmed that daily injections of 0.25 μg T4 or 5 mg of sodium iodide were equivalent in restoring a normal growth rate to thyroidectomized rats, and extended the observations to show that both treatments resulted in small increases in plasma T4 and T3 concentrations (J.-A. Green & A. J. Hulbert, unpublished results). The plasma concentrations of both T4 and T3 following these two treatments, however, were considerably lower than those in the euthyroid controls and thus support the contention that normal growth may not require normal thyroid hormone concentrations. Whether the iodide ion itself influences growth is still unknown.

The influence of hypothyroidism on cell proliferation in the growing rat is manifest by the reduced tissue DNA content, which is most pronounced in those tissues undergoing proliferation (Brasel & Winick, 1970). The stimulatory effects of thyroid hormones on growth are not restricted to the whole organism in that *in vitro* cell proliferation is also stimulated by T4 in cell culture. Both T3 and T4 can stimulate cell growth and mitotic rate of GH3 cells in culture, as well as alter their morphological appearance (Kitagawa *et al.*, 1987). In this respect, it is also of interest that cells in culture have a requirement for polyunsaturated fatty acids for cell division and that non-availability of polyunsaturates can lead to cessation of DNA synthesis and growth (Holley, Baldwin & Kiernan, 1974; Hatten, Horwitz & Burger, 1977; Doi *et al.*, 1978).

Whilst thyroid status influences GH secretion, many of the anabolic and mitogenic effects of GH are mediated by insulin-like growth factor 1 (IGF-1) secreted by the liver into the blood in response to GH. The low plasma GH concentration in hypothyroid young humans is associated with decreased plasma concentrations of IGF-1 and these are elevated following T4 treatment (Chernausek & Turner, 1989). In addition to hepatic production of IGF-1, many other tissues also express IGF-1 mRNA and the IGF-1 peptide, which is believed to have local functions (for review see LeRoith *et al.*, 1995). The actions of IGF-1 are mediated by the IGF-1 cell surface receptors and are further modulated by a complex series of up to six non-receptor IGF binding-proteins (for review see Rechler, 1995). As well as affecting plasma IGF-1 concentrations, thyroid status in humans also influences IGF-1 bioactivity and the concentrations of IGF-binding proteins (Miell *et al.*, 1993).

Whilst many of the growth-retardation effects due to hypothyroidism can be explained by the secondary reduction in hepatic IGF-1 secretion, the inability of GH administration alone to restore normal growth to hypothyroid children (Greenberg *et al.*, 1974) shows that thyroid hormones also stimulate growth by other pathways. This is also demonstrated by the fact that administration of GH and T4 together has a greater effect on the reversal of growth retardation of rats than does GH administration alone (Burstein *et al.*, 1979). Not all thyroid hormone effects on the IGF system in rats are secondary effects mediated by GH (Nanto-Salonen *et al.*, 1993) and the administration of T4 alone to rats is capable of stimulating IGF-1 activity in the absence of GH (Gaspard *et al.*, 1978). Indeed, it has been suggested that there is a feedback loop

regulating peripheral thyroid hormone action involving the GH-IGF axis, in that administration of IGF-1 to rats results in a decrease in TRs (and their respective mRNAs) as well as the activities of two thyroid-hormone-responsive liver enzymes (Pellizas *et al.*, 1998). The physiological relevance of this finding awaits demonstration that the doses of IGF-1 used are relevant to the euthyroid situation in rats.

Although thyroid hormone replacement in hypothyroid individuals restores normal growth, excess thyroid hormone does not increase final body height (Bernal & DeGroot, 1980). For reviews of thyroid hormone effects on bone and mineral metabolism the reader is referred to Auwerx & Bouillon (1986) and Klaushofer *et al.* (1995). At low concentrations, thyroid hormones stimulate the growth and calcification of bone, whilst at high concentrations they inhibit bone growth and stimulate resorption (Ren *et al.*, 1990), and in adult humans, thyrotoxicosis results in bone loss (Fraser *et al.*, 1971). It is thus of interest that both T3 and T4 have biphasic effects on IGF-1 production by rat osteoblastic cells in culture and by fetal rat limb bones. Both these bone cell systems exhibit a basal secretion of IGF-1 that is enhanced by thyroid hormones, however at high concentrations both T3 and T4 dramatically inhibit IGF-1 secretion (Lakatos *et al.*, 1993).

Transplant studies have shown that skeletal and other tissues from fetal rats grow equally well in hypothyroid and euthyroid rats whilst those from juvenile rats show a growth retardation in hypothyroid hosts (Cooke, Yonemura & Nicoll, 1984). The potential importance of thyroid hormones in skeletal development is illustrated by the finding that in serum-free culture conditions, chondrocytes do not mature, but the addition of both T3 and T4 can induce full expression of chondrocyte hypertrophy leading to matrix calcification, the normal process involved in the conversion of cartilage to bone (Alini *et al.*, 1996).

The mode of thyroid hormone action on bone remodelling is not known. Work with bone organ cultures and cultured osteoblasts shows that thyroid hormones increase the production of the prostaglandin PGE_2 and that inhibitors of PGE_2 production diminish the bone resorption caused by both T4 and T3. The fact that T3 binds to osteoblast nuclei from various sources has resulted in the suggestion that thyroid hormones exert their bone resorption effects *via* nuclear receptors. However, if following thyroid hormone treatment the arachidonic acid (20:4; the precursor of PGE_2) content increases in the membranes of bone cells as it does in other cells (e.g. see Table 3), then it is possible that these hormones may initiate their effects on bone cells at the membrane (rather than the nucleus) by resulting in increases in the levels of the precursor of PGE_2. Non-genomic effects on bone cells have been reported in that T3 stimulates the inositol second messenger system in rat bone rudiments, within 30 seconds (Lakatos & Stern, 1991). For a discussion of thyroid effects on bone cell growth and differentiation the reader is referred to Klaushofer *et al.* (1995) and Williams, Robson & Shalet (1998).

A membrane site for thyroid hormone stimulation of cellular growth is compatible with the finding that elevated arachidonic acid (20:4) content of membranes stimulates cell growth and increases mRNA levels of growth-related early response genes (c-fos and Egr-1) in non-bone cell cultures (Danesch, Weber & Sellmayer, 1996). As they are mediated by increased PGE_2 production, such effects were restricted to the n-6 PUFA, arachidonic acid and were not observed following increases in the n-3 PUFAs. Several other cell cultures have also been reported to show increased cellular proliferation following alteration of their membrane n-6 PUFA content (e.g. Holley *et al.*, 1974; Murphy, 1986; Sylvester *et al.*, 1994).

(7) Other effects

Effects to be discussed in this section are those related to: (i) reproduction, (ii) immune and antiviral defence, and (iii) defence against free radicals (i.e. as antioxidants).

In many vertebrate species, reproduction occurs only at certain times of the year and during the remainder of the year such species are refractory to reproduction. European starlings *Sturnus vulgaris* are such seasonal breeders and following thyroidectomy remain in their breeding season indefinitely (Woitkewitsch, 1940). Many bird and mammal species have since been shown to require thyroid hormones for the cessation of reproductive activity in their normal seasonal breeding cycle (for review see Karsch *et al.*, 1995). Experiments with male American tree sparrows *Spizella arborea*, has shown them to be programmed for photoperiodic gonadal growth and separately for post-nuptial moult. Both events are dependent on thyroid hormones and findings suggest that separate neural control circuits are organized early in the breeding season (Wilson & Reinert, 1996). In both this and another bird species, it has been demonstrated that T4 and not T3 is the 'active' thyroid hormone (Pant & Chandola-

Saklani, 1995; Reinert & Wilson, 1997). Experiments with the ewe have shown that thyroid hormones are required for only a short 'window' of time during the breeding season for the development of anoestrus at the end of the breeding period (Thrun *et al.*, 1996; Karsch *et al.*, 1995).

Such thyroid-hormone-dependent seasonal cessation of reproductive activity is mediated by the hypothalamic-pituitary-gonad axis, involving both gonadotropin-releasing hormone (GnRH) neurones and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. The role of the GnRH system in seasonal breeding has been reviewed by Lehman *et al.* (1997). In two seasonal breeding mammal species (sheep and hamsters), an antibody to both TRα1 and TRα2 co-localized with GnRH neurones in the brain (Jansen *et al.*, 1997). However, considering that the effect of thyroid hormones is restricted to a 'window' of time that precedes anoestrus by a considerable period, such nuclear thyroid receptors may not be directly relevant to setting the seasonal breeding timetable. Instead, it is tempting to speculate that the mode of this thyroid hormone effect involves T4-dependent interactions between neurones and astrocytes and laminins in the extracellular matrix (see Leonard & Farwell, 1997). This is likely in view of the fact that there is considerable seasonal plasticity in the degree of synaptic inputs to GnRH neurones, and GnRH neurones differ from neighbouring neurones in that they are almost completely surrounded by glial processes from adjacent astrocytes (Lehman *et al.*, 1997). Experiments examining the relative potencies of rT3, T3 and T4 in such situations will probably give additional insight into the mechanisms involved.

Hypothyroidism has no influence on testicular development in prenatal rats but postnatal hypothyroidism results in reduced levels of gonadotropins and delays pubertal spermatogenesis (Francavilla *et al.*, 1991). In the adult female rat, hypothyroidism results in dramatically extended oestrus cycles (Evans *et al.*, 1966). Whether this is an effect on the gonadotropin system or an effect at the ovarian level is not known. Thyroid hormones are known to influence ovarian cells. For example, T3 exposure influences steroid (androgen, estradiol and progesterone) production by porcine ovarian follicle cells. The effects are complex in that they depend on the particular type of follicle cell, the stage of follicular development and also demonstrate interactions with human chorionic gonadotropin exposure (Gregoraszczuk & Skalka, 1996). Exposure of cultured human granulosa cells to T3 stimulates the *in vitro* production of tissue inhibitor of metalloproteinases 1 (TIMP-1) which is suggested to be involved in ovulation, luteal development and regression as well as trophoblast invasion (Goldman *et al.*, 1997). T3 has also been suggested to be a negative modulator of steroidogenic function in the avian adrenal gland (Carsia *et al.*, 1997).

Thyroid status has limited influence on the immune system. For example, neither hypothyroidism nor hyperthyroidism had significant effects on the number of blood mononuclear cells or immune function in the rat or guinea pig (Wall, Twohig & Chartier, 1981). At supraphysiological concentrations, thyroid hormones alter the response of lymphocytes to mitogens but have no such effects at physiological levels (Ong, Malkin & Malkin, 1986). Thyroid hormones at physiological concentrations do however potentiate the antiviral action of interferon γ in cultured human cells, and T4 is approximately ten times more potent than T3 (Lin *et al.*, 1994). Thyroid hormone itself is not antiviral and various analogues of thyroid hormones lack the ability to potentiate interferon activity. It has been suggested that there are two pathways for such an effect of thyroid hormones, one dependent on, and the other independent of protein synthesis (Lin *et al.*, 1996). The protein-synthesis-dependent pathway is blocked by inhibitors of protein kinases C and A and appears to be independent of thyroid nuclear receptors (H.-Y. Lin *et al.*, 1997).

Although aerobic organisms cannot exist without oxygen, the reductive nature of the cellular environment provides ample opportunities for the creation of reactive oxygen species (ROS) which are extremely dangerous to living systems in that they can damage proteins, lipids, carbohydrates and nucleic acids. This has been called the 'oxygen paradox' (for review see Davies, 1995). Thus, aerobic organisms both generate and accumulate a variety of water- and lipid-soluble antioxidant compounds, as well as producing a series of antioxidant enzymes, to intercept and inactivate ROS. Membrane phospholipids are continually subject to such oxidative damage and lipid peroxidation is an autocatalytic chain of reactions initiated at the double bond of an unsaturated acyl chain. Phospholipids are more prone to peroxidation than triglycerides, with the highly polyunsaturated acyl chains (e.g. 20:4 and 22:6) being the most sensitive to damage (e.g. Catala & Cerruti, 1997). It is estimated that approximately 1% of daily oxygen consumption goes to mitochondrial ROS generation.

Another major source are phagocytic cells which use an NADPH oxidase system to produce ROS as a weapon against invading microrganisms. Such cells only produce ROS in large amounts when they have been activated to phagocytose (Davies, 1995).

Thyroid hormones themselves also pose a paradox. This 'thyroid hormone paradox' is that they both promote damage by ROS, by stimulating aerobic metabolic activity, but they also act as antioxidants themselves, as well as influencing other antioxidant defences.

Hyperthyroidism increases lipid peroxidation in young, old and very old rats and hypothyroidism results in lower levels of lipid peroxidation in young rats (Mooradian *et al.*, 1994). One study reports that hypothyroidism in rats significantly decreases lipid peroxidation in skeletal muscle but not thymus, spleen or lymph nodes (Pereira *et al.*, 1994), whilst another reports that it results in no reduction in lipid peroxidation in liver, cardiac or skeletal muscle (Venditti *et al.*, 1997*a*). In rats, hyperthyroidism results in increased levels of lipid peroxidation products in liver, heart, soleus muscle and lymph nodes (Asayama *et al.*, 1989; Pereira *et al.*, 1994; Venditti *et al.*, 1997*a*). Higher levels of mitochondrial enzymes and increased ROS production by submitochondrial particles has also been reported for hyperthyroid rats (Fernandez & Videla, 1993).

Thyroid-induced increases in lipid peroxidation are not restricted to enhanced mitochondrial activity. For example, in rats T3 increases superoxide production, NADPH oxidase activity and lipid peroxidation in hepatic microsomes (Fernandez *et al.*, 1985). Polymorphonuclear leucocytes from hyperthyroid rats have an enhanced capacity to produce superoxide when stimulated *in vitro* and this seems primarily related to thyroid-hormone-enhanced NADPH oxidase activity (Fernandez & Videla, 1995). A similar effect has been observed in phagocytes from hyperthyroid humans (Videla *et al.*, 1993). Both T4 and T3 can also stimulate, *in vitro*, the activity of myeloperoxidase isolated from human leukocytes (Van Zyl, Basson & Van der Walt, 1989). Low-density lipoproteins isolated from hyperthyroid humans also show an elevated lipid peroxidation compared to those from euthyroid individuals, intriguingly as do those from hypothyroid individuals (Costantini *et al.*, 1998). These results suggest that, at least in humans, the euthyroid condition represents that which results in the minimal peroxidative damage to low-density lipoproteins. Whether this also applies to other molecules capable of oxidative damage is not known.

Cells utilize a number of antioxidant scavengers and enzymes as a defence against damage by ROS. Vitamin E is a major membrane antioxidant whilst vitamin C is a major aqueous antioxidant. Other membrane-bound antioxidant compounds include β-carotene, ubiquinone and, as will be discussed shortly, possibly thyroid hormones. Antioxidant enzymes include superoxide dismutase (SOD), glutathione peroxidases and catalases (Davies, 1995). The influence of thyroid status on antioxidant defences is mixed and there appears to be no consistent pattern. Some studies report that hyperthyroidism increases vitamin E content of cardiac muscle (Mano *et al.*, 1995; Venditti *et al.*, 1997*a*) whilst others show no effect (Asayama *et al.*, 1989). Hyperthyroidism increases glutathione levels in erythrocytes and serum (Morini *et al.*, 1991; Seven *et al.*, 1996) but decreases glutathione levels in rat liver (Morini *et al.*, 1991). During hyperthyroidism, glutathione peroxidase is elevated in liver, erythrocytes, spleen and skeletal muscle (Morini *et al.*, 1991; Seven *et al.*, 1996; Pereira *et al.*, 1994; Fernandez & Videla, 1995) diminished in thymus, heart and skeletal muscle (Seven *et al.*, 1996; Pereira *et al.*, 1994; Asayama *et al.*, 1989; Fernandez & Videla, 1995; Mano *et al.*, 1995) and unaffected in liver and heart (Fernandez & Videla, 1995). Whilst in hypothyroidism it has been reported as both increased in cardiac and skeletal muscle (Fernandez & Videla, 1995; Mano *et al.*, 1995) and reduced in thymus and skeletal muscle (Venditti *et al.*, 1997*a*; Pereira *et al.*, 1994). Glutathione reductase activity appears relatively unaffected by thyroid status (Venditti *et al.*, 1997*a*) although it has been reported to be elevated in liver during hyperthyroidism (Morini *et al.*, 1991). Catalase activity is reported both to increase in skeletal muscle (Pereira *et al.*, 1994) and decrease in thymus, spleen, cardiac and skeletal muscle (Pereira *et al.*, 1994; Asayama *et al.*, 1989; Seven *et al.*, 1996) during hyperthyroidism. The activity of Cu^{2+},Zn^{2+}-SOD is also reported to be increased in thymus, leucocytes and skeletal muscle (Pereira *et al.*, 1994) but decreased in cardiac and skeletal muscle (Asayama *et al.*, 1989) whilst Mn^{2+}-SOD is reported to be increased in leucocytes, thymus, spleen, cardiac and skeletal muscle (Pereira *et al.*, 1994; Asayama *et al.*, 1989) during hyperthyroidism. No general pattern can be discerned from the diverse responses of antioxidant defences to hyperthyroidism. Most studies involving hypothyroidism report no significant influence on levels of either antioxidant scavengers or enzymes.

The question as to whether thyroid hormones

themselves can act as antioxidant compounds was first raised after De Caro (1933) observed that T4 can decrease oxygen uptake by solutions of unsaturated fatty acids. It was later shown that T4 could also prevent lipid peroxidation in isolated erythrocytes (Bunyan *et al.*, 1960) and liver homogenates (Bunyan *et al.*, 1961), and that T4, T3 and 3,5-T2 (but not the non-iodinated thyronine) could inhibit lipid peroxidation in isolated rat liver mitochondria as well as dramatically inhibiting carbon tetrachloride accelerated lipid peroxidation (Cash *et al.*, 1967). As with many early studies, the T4 concentrations used in these experiments were high and non-physiological, the lowest being 1 μM. Wynn (1968*b*) used an *in vitro* system to show that Fe^{2+} could activate lipid peroxidation of rat liver microsomes and that T4 could terminate such lipid peroxidation by acting as an antioxidant. The lowest T4 concentration examined was 100 nM, and at this concentration, T4, rT3 and T3 all showed antioxidant activity (in decreasing intensity) and their antioxidant abilities were greater than that of either vitamin E or ascorbic acid in the same system. He also showed that pharmacological doses of T4 could reduce *in vivo* the peroxide content of epididymal fat pads in the rat (Wynn, 1968*a*). Because of its relative metabolic inertness such a tissue is unlikely to have had significant thyroid-hormone-induced peroxidation. In these lipid peroxidation experiments, T4 was degraded when exerting its antioxidant effects. This was consistent with the finding that the *in vivo* rate of T4 degradation seemed to be related to its clinical effect (Galton & Ingbar, 1962). Lipid peroxidation can also be catalyzed *in vitro* by hemoglobin, and iodothyronines inhibit such peroxidation, and are more potent antioxidants in these situations than vitamin E, glutathione or ascorbic acid (Tseng & Latham, 1984).

The physiological importance of the antioxidant properties of thyroid hormones was questioned when the antioxidant capacities of both T3 and T4 in retarding the auto-oxidation of rat brain homogenate and free-radical-mediated oxidation of rat erythrocyte membranes was evaluated and found to be very low (1–2%) at hormonal concentrations of 50 nM but substantial at micromolar hormonal levels (Faure, Lissi & Videla, 1991).

However, a more recent report of T4 and T3 inhibition of ROS generation by activated human neutrophils (Antipenko & Antipenko, 1994) raises questions with respect to the possible physiological importance of the antioxidant properties of the thyroid hormones. These findings have been reproduced in Fig. 10. When human neutrophils are stimulated by pyrogenal-lipopolysaccharide from the *Salmonella typhi* cell wall, they initiate ROS generation that can be monitored by a chemiluminescence assay. Thyroid hormones at high concentrations (5 μM) inhibit this chemiluminescence as does another powerful antioxidant, ionol (4-methyl-2,6-di-isobutyl phenol). This inhibition decreases to approximately half as thyroid hormone concentrations decrease to 1 nM. Surprisingly, when T4 and T3 concentrations are further decreased to 10 pM and 0.1 pM, chemiluminescence inhibition increases. Such a bimodal response suggests that two separate processes are operating. The effect was only observed if T4 and T3 were added prior to stimulation of ROS production and only when the ROS generation stimulus was pyrogenal and not when it was 4β-phorbol 12-myristate 13-acetate (PMA). Whilst T4 and T3 showed a bimodal antioxidant response, the powerful antioxidant, ionol did not. These results suggest that T4 and T3, at very low concentrations, have effects that influence ROS production. They are more potent than ionol, which is in turn is a more potent antioxidant than vitamin E. These authors also discuss the proposal that thyroid hormones are involved in other cellular repair mechanisms.

That thyroid hormones possibly have antioxidant activity is also illustrated by the finding that although resting neutrophils exhibit negligible deiodination of both T4 and T3, when they are stimulated to phagocytose (and ROS production is consequently activated), the deiodination of both T4 and T3 dramatically increases severalfold. Neutrophils from individuals with chronic granulomatous disease, which are deficient in the capacity to produce ROS, degrade T4 and T3 poorly during phagocytosis (Klebanoff & Green, 1973).

Examination of the potential role of thyroid hormones as potent natural membrane antioxidants also deserves attention in light of other observations. As can be seen from Fig. 4, a strong correlation has been observed in rats between the phospholipid content of tissues and their T4 content. Analysis of this relationship, assuming that all T4 is associated with membranes, suggests a molar ratio of T4:phospholipid of approximately 1:1000. This is similar to the molar ratio of vitamin E:lipid observed in biological membranes which is of the order of 1:2000–3000 (Packer & Landvik, 1989). Whilst obviously not all thyroid hormone molecules are associated with membranes, measurements in human erythrocytes suggest that approximately half of

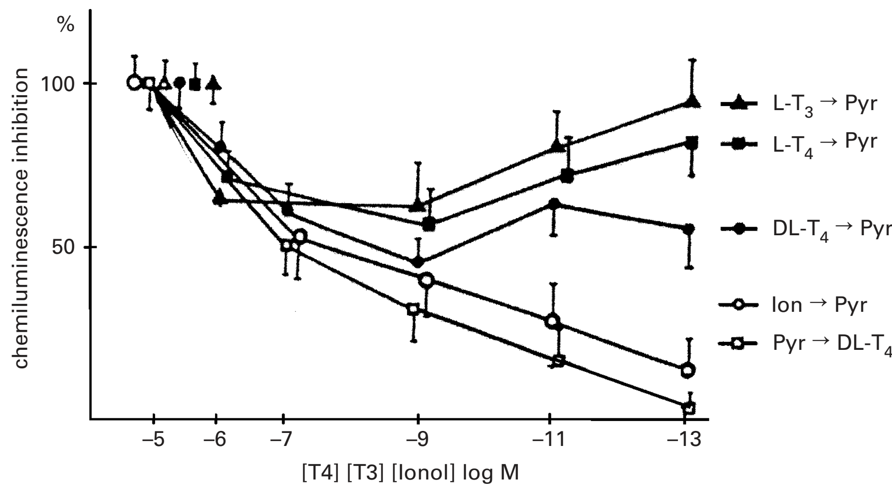

Fig. 10. The influence of the concentration of thyroid hormones (T4 & T3) and ionol (Ion) on the release of reactive oxygen species (monitored as chemiluminescence) by human neutrophils activated by pyrogenal-lipopolysaccharide (Pyr) from *Salmonella typhi* cell wall. The symbols on the right of the figure indicate the sequence of addition. Reprinted with permission from Antipenko & Antipenko (1994).

cellular thyroid hormone molecules (34% of T4 and 60% of T3) are associated with membranes (Bregengaard *et al.*, 1989). As the human erythrocytes lack intracellular membranes and in this particular study they were washed several times before measurement, these are likely to be minimum values.

Another reason why this area is worthy of careful examination is that the resting oxygen consumption of mammals is proportional to approximately the 0.75 power of body mass (Kleiber, 1961). This is the same allometric exponent observed for whole-mammal ethane exhalation rate, which is indicative of lipid peroxidation (Topp *et al.*, 1995) and for the degradation rates of T4 and T3 in mammals (Tomasi, 1991). These findings of similar allometric exponents for these three processes means that, in mammals, irrespective of metabolic activity, there is a relatively constant molar ratio between oxygen consumption, phospholipid peroxidation and deiodination of T4 and T3.

IV. THYROID HORMONES AND VERTEBRATE DEVELOPMENT

(1) Thyroid axis during vertebrate development

The influence of thyroid hormones on vertebrate development was first demonstrated by Gudernatsch (1912) who showed that when tadpoles were fed desiccated horse thyroid tissue they precociously metamorphosed into frogs. Since that time it has become obvious that the thyroid is also involved in normal development of vertebrates, ranging from fish to mammals.

Indeed, thyroid hormones may be necessary from conception for normal development. Interestingly, iodine deficiency in female rats results in a dramatic decrease in the size of their young even when such iodine deficiency is ceased at the time they conceive their young (Sunitha, Udaykumar & Raghunath, 1997). Pharoah, Buttfield & Hetzel (1971) have shown that iodized oil must be given prior to conception to iodine-deficient mothers to prevent neurological damage in their children. In sheep, maternal thyroidectomy prior to conception results in neurological damage (Potter *et al.*, 1986). Although the plasma concentrations of thyroid hormones are relatively constant throughout adult life of most vertebrates, all vertebrate classes examined to date, during their early development, have a surge of plasma T4 (and T3) to levels that exceed those found in the adult plasma.

Thyroid hormones are found in the eggs of fish (Tagawa & Hirano, 1987; Leatherland *et al.*, 1989) and birds (Sechman & Bobeck, 1988; Prati, Calvo & Morreale de Escobar, 1992; Wilson & McNabb, 1997). The detection of both T4 and T3 in amphibian embryos one day after fertilization (at, respectively, approximately 0.6 and approximately 4.7 pmol g^{-1}) suggests that they are also present in amphibian eggs (Weber *et al.*, 1994). Nothing is known concerning reptilian eggs. Because mam-

malian eggs remain in the mothers body, it is also likely that mammalian eggs contain thyroid hormones. In fish eggs, the T4 content can either exceed that of T3 (e.g. Leatherland *et al*., 1989) or be less than the T3 content (e.g Yamano & Miwa, 1998). Partial depletion of both T4 and T3 in the eggs of the Medaka fish *Oryzias latipes* had no discernable effects on either hatching, survival or development of the young fish (Tagawa & Hirano, 1991). In the amphibian embryo, T3 concentration exceeded T4 concentration (Weber *et al*., 1994) whilst the opposite was the case for avian eggs (Sechman & Bobeck, 1988; Prati *et al*., 1992; Wilson & McNabb, 1997). The deposition of thyroid hormones in avian eggs and their role has recently been reviewed by McNabb & Wilson (1997).

There is a surge of T4 concentration during metamorphosis in the flounder *Paralichthys olivaceus* (Miwa *et al*., 1988). In tilapia fish *Oreochromis mossambicus*, there is a surge of T4 concentration which corresponds in time to a morphogenic change in body shape and is followed by an increase in T3 concentration (Okimoto, Weber & Grau., 1993). Inhibition of thyroid function with goitrogens has been shown to inhibit, and exogenous T4 to stimulate, the metamorphosis of larvae to juvenile fish in the flounder (Miwa & Inui, 1987), and the zebrafish *Danio rerio* (Brown, 1997), whilst both exogenous T4 and T3 promote early metamorphosis in grouper *Epinephelus coioides* larvae (de Jesus, Toledo & Simpas, 1998). A developmental T4 surge has also been observed in the larvae of black sea bream (Tanaka *et al*., 1991) and red sea bream *Pagrus major* (Kimura *et al*., 1992).

Juvenile salmonid fish undergo a parr-smolt transformation that prepares them for the impending sea-water phase of their life cycle, and during this process plasma T4 levels temporarily increase fourfold followed by a smaller T3 increase (Dickhoff, Darling & Gorbman, 1982; Specker *et al*., 1984). In coho salmon *Oncorhynchus kisutch*, a year before the parr-smolt change (which takes place in the second year of freshwater life) there is a small surge in T4 concentration followed by a small increase in plasma T3 levels. Whether this has any developmental importance is unknown but it has been suggested that it may be the basis for 'phase differentiation' measured in other salmon species (Dickhoff *et al*., 1982).

Lampreys are modern representatives of the earliest vertebrates, the jawless agnathan fish, and exhibit metamorphosis. In this group, plasma levels of thyroid hormones increase during the larval period and metamorphosis is associated with a dramatic decrease in plasma thyroid hormone levels. The larvae lack a follicular thyroid gland but possess instead an endostyle (a subpharyngeal gland) that develops into a follicular thyroid in the adult. In premetamorphic lampreys, inhibition of thyroid function induces early metamorphosis whilst exogenous thyroid hormones inhibit metamorphosis (Manzon, Eales & Youson, 1998). The lampreys appear to be unique among vertebrates in the relationship between thyroid function and metamorphosis, whether they represent the original vertebrate condition or a derived condition is unknown. The other extant agnathans, the hagfishes, which are believed to be phylogenetically older than lampreys, do not exhibit metamorphosis and possess a follicular thyroid gland. The role of the thyroid in lamprey metamorphosis has been recently reviewed by Youson (1997).

Although earlier studies suggested that thyroid hormones may not be present in the plasma of premetamorphic amphibians, recent work has shown that both T4 and T3 are measurable in the tissues of amphibian larvae from the time of hatching (Gancedo *et al*., 1997). In amphibians, there is a developmental surge of T4 and T3 concentration that peaks at metamorphic climax (Mondou & Kaltenbach, 1979; Suzuki & Suzuki, 1981; Weber *et al*., 1994). In *Rana catesbeiana*, the T4 surge precedes the T3 surge (Suzuki & Suzuki, 1981) and there is a surge in transthyretin (TTR) mRNA levels in liver during metamorphosis which is absent in the adult. Transthyretin mRNA was not detectable in the tadpole choroid plexus (Yamauchi *et al*., 1998).

Mexican axolotls are neotenous amphibians that rarely undergo metamorphosis. Juvenile axolotls have no detectable thyroid hormones in their plasma whilst adults have no detectable T3 and only low plasma levels of T4, and thus do not appear to have such developmental surges of thyroid hormones (Galton, 1992*b*). However if they are given an exogenous T4 surge they will undergo metamorphic changes (e.g. Brown, 1997).

The egg, especially the yolk, is the source of T4 and T3 found in the tissues of the avian embryo prior to the onset of thyroid function (Prati *et al*., 1992; Wilson & McNabb, 1997). Accumulation of T3 is especially high in the brain and is much greater than inferred from measurement of plasma levels, and in this respect the chicken embryo in similar to the rat fetus (Prati *et al*., 1992). Transthyretin mRNA is produced by the chick embryo liver well before the development of the thyroid gland and rapidly rises

to adult levels, whilst the chick embryo's choroid plexus also produces TTR mRNA during incubation but at levels lower than that found in the adult chicken's choroid plexus (Southwell *et al*., 1991). The role of the unidirectional secretion of TTR into cerebrospinal fluid as a brain thyroid hormone pump may be important in the accumulation of thyroid hormones by the avian embryo's brain.

In the chicken, there is a developmental surge in plasma T4 concentration that peaks just before hatching, whilst the T3 level peaks at the time of hatching and remains high thereafter (Darras *et al*., 1992). In the precocial Japanese quail *Coturnix japonica*, there is a developmental surge of both T4 and T3 levels around the time of hatching, this surge in hormone concentration is relatively brief and manifest in both the total and the free plasma hormone levels (McNabb, Lyons & Hughes, 1984). In the altricial ring doves *Streptopelia risoria*, starlings *Sturnus vulgaris* and redwing blackbirds *Agelaius phoeniceus* there is a more extended developmental surge of plasma T4 levels that begins after hatching and lasts for approximately the first three weeks of post-hatching life (McNabb & Olson, 1996). In ring doves, although total plasma T4 concentration does not demonstrate a pronounced peak, there is an eightfold increase in free T4 levels peaking at approximately day 14 after hatching, that is suggested to be due to displacement from plasma protein binding (McNabb, 1988).

To my knowledge, nothing is known of the developmental profile of thyroid hormones in reptiles or the egg-laying mammals, the monotremes. In the marsupials, the young are born at a very immature phase and undergo a large amount of development in the pouch of the mother. Indeed, it is near the time of pouch exit, rather than birth, that the physiological development of marsupial young is similar to that of newborn eutherian mammals. Marsupial pouch young have been likened to 'exteriorised embryos' and have been proposed as good model systems in which to study mammalian development (see Tyndale-Biscoe & Janssens, 1988). In the tammar wallaby *Macropus eugenii* (Fig. 11) there is a developmental surge of T4 concentration that precedes a T3 surge. In very early pouch young (well before histological development of the thyroid gland), plasma T4 concentration is similar to adult levels, rising to a peak at approximately 160 days of age before returning to adult levels. By contrast, plasma T3 levels in newborn are considerably lower than adult levels and peak later, at approximately 220 days of age, which is after initial pouch exit in this species. The developmental profiles for T4 and T3 are similar for both total and free hormone concentrations. Plasma rT3 levels are higher (and more variable) during pouch life than in adults and show a peak at the same time as the T4 peak. Both liver and kidney show no 5′ deiodination activity at birth but such enzymatic abilities increase during pouch life to reach adult levels shortly after the time of pouch exit (Janssens *et al*., 1990). A similar developmental surge in levels of thyroid hormones has been recorded for another marsupial, the brushtail possum *Trichosurus vulpecula* (Buaboocha & Gemmell, 1995).

Eutherian mammals similarly show developmental surges in T4 and T3 levels. In the rat, both T4 and T3 are found in the embryo prior to onset of fetal thyroid function (Obregon *et al*., 1984) and maternal hypothyroidism at this time results in reduced tissue levels of both T4 and T3 in the fetus, which for some tissues persists throughout gestation (Morreale de Escobar *et al*., 1985). The surge in plasma T4 levels precedes the increase in plasma T3 concentration in the rat (Morreale de Escobar, Obregon & Escobar del Rey, 1987), the horse (Irvine & Evans, 1975), the ferret (Kastner, Kastner & Apfelbach, 1987) and the lamb (Wrutniak, Cabello & Bosc, 1985). In the rat, which is an altricial eutherian, these hormone surges commence before birth but predominantly occur in the first postnatal weeks, whilst in both the sheep and humans, the thyroid hormone surges are prenatal.

Summarizing the accumulated data for vertebrates, there is a severalfold increase in plasma T4 concentration during early vertebrate development which is either followed by, or coincident with, an increase in plasma T3 levels. The plasma T4 surge is more prevalent in vertebrates than a developmental surge in plasma T3 levels.

Deiodinase activity also varies during vertebrate development. In larval lampreys, outer ring (5′) deiodination activity is high in the intestine and liver and low in kidney and muscle, whilst in adult lampreys the intestine, kidney and muscle retain 5′-deiodination activity but it is completely absent from adult liver tissue (Eales *et al*., 1997). Inner ring 5-deiodination activity was not detected in larval tissues but was observed in adult intestine and, to a lesser extent, in adult kidney (Eales *et al*., 1997). Manipulation of thyroid status in larval lampreys, which influenced the timing of metamorphosis, had no influence on intestinal 5′-deiodinase activity (Manzon *et al*., 1998).

In salmon, during the parr-smolt transformation,

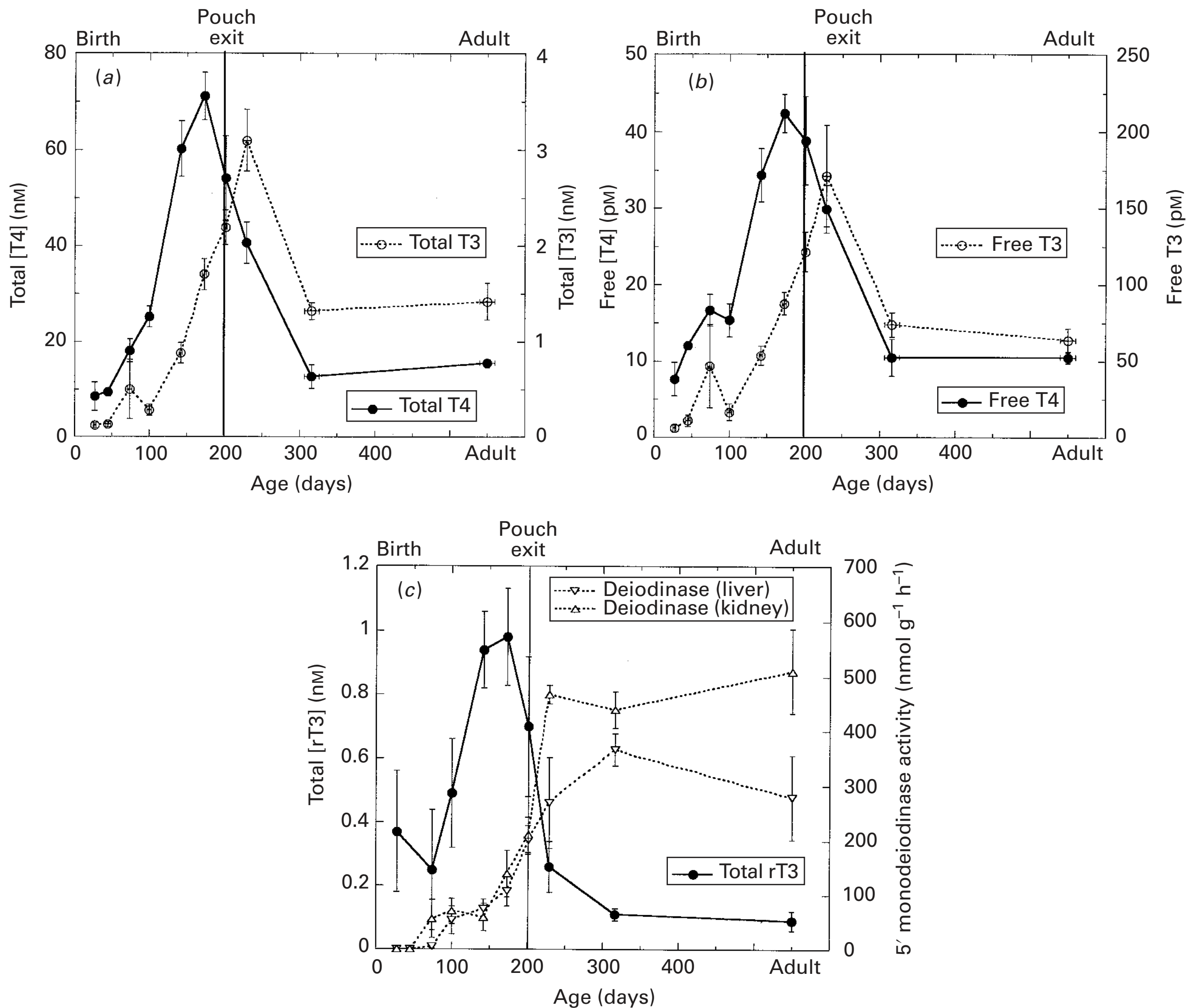

Fig. 11. Thyroid hormone levels and tissue deiodination activity during development of the tammar wallaby Macropus eugenii. (A) The developmental profiles for total plasma T4 and T3 concentrations. (B) The developmental profiles for free plasma T4 and T3 concentrations. (C) The developmental profile for total plasma rT3 concentration and the developmental profile of the in vitro 5′ monodeiodination activity of liver and kidney. In all graphs, the time of initial pouch exit is marked. Young wallabies are at a stage physiologically similar to newborn eutherian mammals at this time. Reproduced using the data of Janssens *et al.* (1990). Values are means +S.E.M. ($N = 2$–8).

changes in T4 production, distribution and metabolism are relatively independent of being in a freshwater or salt-water environment (Specker *et al.*, 1984). Tissue thyroid hormone levels do not echo plasma concentrations during this period (Specker, Brown & Bern, 1992). During the parr-smolt transformation, there is enhanced outer ring 5′-deiodination activity in liver and heart, but in gill and skeletal muscle the relatively low deiodinase activity remains unchanged. Plasma T3 levels are correlated with liver 5′-deiodination activity and brain 5′-deiodination activity increases at the end of the transformation. Inner ring 5-deiodination activity is high in brain tissue, as well as heart and gill, but is low in liver and muscle (Morin, Hara & Eales, 1993; Eales *et al.*, 1993). These studies suggest that tissue deiodinase activities are probably important in determining different developmental profiles for intracellular thyroid hormones in individual tissues during the parr-smolt transformation in salmonid fish.

Galton & Cohen (1980) observed negligible *in vivo* conversion of T4 to T3 in premetamorphic tadpoles yet both exogenous T4 and T3 had effects in these premetamorphic tadpoles. Later it was demonstrated that outer ring 5′-deiodinase activity was

absent in liver, heart, kidney and tail during premetamorphosis but was present in skin and exhibited minimal activity in brain and intestine whilst inner ring 5-deiodinase activity was detectable in most tissues during premetamorphosis, being highest in liver, intestine and kidney. At metamorphic climax, induced by T4 exposure, 5′-deiodinase activity dramatically increased in skin and intestine and was present in tail but remained absent in liver, heart and kidney, whilst 5-deiodinase activity became barely detectable (Galton & Hiebert, 1988). Galton (1988) examined tissue deiodinase activities during spontaneous metamorphosis and extended measurements to include prometamorphosis and adult tissues. She found that 5′-deiodinase activity was highest in skin and intestine and negligible in other tissues. In prometamorphic tadpoles, deiodinase activity was comparable to that in premetamorphic tadpoles and it remained high in adult tissues. In the adult frog, whilst 5′-deiodinase activity was only observed in intestine and skin, inner ring 5-deiodinase activity was present in all tissues examined. Unexpectedly, in view of the observation that 5-deiodinase activity decreases during metamorphic climax, it has been shown that 5-deiodinase (D3) gene expression and D3 enzyme activity is increased by exposure to T3 (Becker *et al.*, 1995). The measurement of both 5′-deiodinase (D2) and 5-deiodinase (D3) enzyme activity in a variety of tissues during metamorphosis in the amphibian *Rana catesbeiana* (Becker *et al.*, 1997) has strongly supported the concept that coordinated development of different tissues during the developmental surge of extracellular T4 levels could be determined by specific ontogenetic profiles for the deiodinases in different tissues. In this excellent study, the authors showed that some tissues (e.g. hindlimb) have high D2 deiodinase activity prior to, and negligible activity after metamorphic climax, whilst other tissues (e.g. tail, intestine, forelimb) had the opposite profile and still other tissues (e.g. skin, eye) had a relatively constant activity throughout the various stages of amphibian development. The activity of the inner ring D3 deiodinase also showed a developmental profile that differed between tissues. Thus, by tissue-specific changes in deiodinase activities, the common developmental surge in extracellular T4 levels can be modified in individual tissues. Prior to metamorphic climax there is a decline in liver 5-deiodinase activity whilst after metamorphic climax there is an increase in 5′-deiodinase activity in skin and intestinal tissue (Galton, 1992*a*). There is a shift in the preponderance of inner ring 5-deiodination prior to metamorphic climax to predominantly outer ring 5′-deiodination after climax.

The ontogeny of deiodination in birds has been reviewed by McNabb (1988). In the precocial Japanese quail, at the time of the T4 surge around the time of hatching there is also a dramatic increase in outer ring 5′-deiodination in the liver, which is initially due an small increase in D2-type deiodinase but is rapidly replaced by a much larger increase in D1-type deiodinase activity. After its initial surge in activity, the D1 deiodinase declines to adult levels during the first few days after hatching. In the altricial ring dove, hepatic 5′-deiodinase activity is relatively constant in embryos and for a few days after hatching after which it declines in activity. The decline in 5′-deiodinase activity after hatching in this species is the opposite of changes in the serum T3/T4 ratio which shows an increase during this period (McNabb, 1988) This is probably due to the even more dramatic decline in 5-deiodination which will remove T3 formed from T4. During the last week of incubation, the chick embryo liver shows a substantial increase in outer ring 5′-deiodinase activity, whilst there is an even more dramatic decrease in inner ring 5-deiodinase activity over the same period (Galton & Hiebert, 1987). Others have confirmed that in the chick embryo, D1 deiodinase activity increases whilst D3 deiodinase activity decreases during the last days of incubation (Van der Geyten *et al.*, 1997). A decline in 5′-deiodinase activity following hatching is also reported for the chicken and is unlike the situation after birth in some mammals. Although hepatic 5′-deiodination decreases after hatching in the chicken, the decrease in 5-deiodination is even greater and begins approximately 4–5 days prior to hatching. In the newly hatched chicken, hepatic 5-deiodination is negligible (Darras *et al.*, 1992). The deiodinase activities of avian tissues other than liver, and their importance, is relatively unknown. In the chicken, kidney deiodinase activity is less than that found in liver and is relatively constant before and after hatching (Darras *et al.*, 1992). The chicken brain is reported to have both D2 and D3 deiodinases and the deiodinase system in birds is reported to be similar to that in mammals (Kuhn, Mol & Darras, 1993).

During early pouch life in the marsupial *Macropus eugenii* neither liver nor kidney demonstrate any *in vitro* 5′-deiodinase activity. However, at approximately the time of the plasma T4 surge, *in vitro* 5′-deiodinase activity increases in both liver and kidney to levels found in the adult tissues (Fig. 11). The fact

that significant amounts of rT3 are measured in pouch young plasma prior to this period suggests that 5-deiodination activity is present from a very early developmental stage. The decline in rT3 towards the end of pouch life supports the conclusion that around the time of the developmental T4 surge there is a shift in emphasis from inner ring to outer ring deiodination in this marsupial species (Janssens *et al.*, 1990).

In the rat, 5′-deiodinase activity is absent in liver and intestine until day 18 of fetal life, after which it appears in both tissues and gradually increases in activity. However, whereas 5′-deiodinase activity is much lower in the fetal rat than the adult mother, the opposite is the case for 5-deiodination which is especially high in fetal intestine and brain compared to adult intestine and brain and of approximately similar activity in fetal and adult rat liver (Galton, McCarthy & St. Germain, 1991*a*). In the rat, there is fourfold increase in the activity of brain D2 5′-deiodinase between gestational days 17 and 22 (birth) that is correlated with an increase in brain T3 content. Inhibition of thyroid function during this period results in significant increases in D2 activity (Ruiz de Ona *et al.*, 1988).

Selenium deficiency in rats (which causes a near-complete loss of hepatic D1 5′-deiodinase in adults) does not interfere with the postnatal plasma T3 surge, and it has thus been suggested that peripheral deiodination is not important in this T3 surge but that it may predominantly be due to intrathyroidal deiodination of T4 (Chanoine *et al.*, 1993*b*). In sheep, there are increases in both D1 and D3 deiodinase activities after the T4 surge which begins before birth. The dramatic decrease in rT3 concentration and the increase in T3 concentration in sheep plasma at birth demonstrates that this species too shows a switch from predominantly inner ring to outer ring deiodination at this time (Fisher, Polk & Wu, 1994). In humans, the increase in both T4 and rT3 levels during the second half of gestation precedes the increase in T3 levels which does not commence until approximately three-quarters of the way through gestation. This suggests that a similar switch occurs in humans (Fisher et al., 1977).

The switch over from inner ring to outer ring deiodination during development appears to be a general finding for many higher vertebrates. Whilst deiodinase activity has been predominantly studied in liver tissue, the finding of tissue-specific profiles in intracellular deiodinase activities during development in amphibians, together with the finding of high intestinal deiodinase activity in the rat fetus but its relative absence in adult rat intestine, and the importance of tissue deiodination for determining normal tissue T4 and T3 levels in the adult rat, suggests that there may be tissue-specific deiodination profiles during the development of vertebrates. Indeed, in the rat, the development of 5′-deiodinase activity has different ontogenetic patterns in different tissues which consequently affect the ontogenetic profiles for tissue T3 (Obregon *et al.*, 1989).

The concentrations of the two types of nuclear thyroid receptors and their respective mRNAs also vary during vertebrate development. The differential tissue expression of these receptors has been elegantly demonstrated with *in situ* hybridization of tissues from the developing flounder (Yamano & Miwa, 1998). This study showed that virtually every tissue expressed either an α- or β-type TR gene, and that both the intensity and the tissue distribution of the two types of TR mRNA varied. At metamorphic climax, the TRα mRNA concentration was greatest in skeletal muscle and digestive epithelium, whereas TRβ mRNA was strongest in cartilage cells and presumed osteoblasts, as well as in the myosepta which separate the myotomes. At a whole-body level, TRα mRNA is more prevalent in the larval flounder than TRβ mRNA. During flounder metamorphosis TRα mRNA concentration increases and peaks at climax, after which it decreases dramatically in the postclimax flounder. TRβ mRNA concentration increases during metamorphic climax and peaks after the TRα mRNA peak, after which it remains high. Unlike the situation in the larval flounder, in the post-climax flounder TRβ mRNA is more prevalent than TRα mRNA (Yamano & Miwa, 1998).

It has been reported that the TRα gene is expressed in eggs and embryonic stages that precede the maturation of the thyroid gland in *Xenopus laevis* and that TRα mRNA levels increase dramatically before metamorphic climax (Banker, Bigler & Eisenman, 1991). That it may have some effects at this early stage is suggested by the fact that precocious increase in synthesis of the α receptor leads to hormone-dependent developmental abnormalities in *Xenopus laevis* embryos (Old *et al.*, 1992). In the larval amphibian, at the whole-animal level, TRα mRNA predominates over TRβ mRNA and reaches high levels prior to metamorphic climax. The concentration of TRβ mRNA increases just prior to metamorphic climax and reaches its peak level at climax after which the mRNA for both types of thyroid receptors decreases (Yaoita & Brown,

1990). There is also differential expression of TRs in amphibian tissues, in that the nucleated amphibian erythrocytes express the gene for TRα but not TRβ (Schneider, Davey & Galton, 1993), whilst transcripts of the TRβ gene have been detected in various tissues (tail, skin, kidney, leg, heart and intestine) of premetamorphic tadpoles but not in their erythrocytes (Davey, Schneider & Galton, 1994). In both cases, mRNA levels are highest at metamorphic climax and are decreased in the adult frog. Furthermore, the amount of transcript for both types of receptors increases in the presence of T3. Kanamori & Brown (1992) have shown that TRβ mRNA is absent in unfertilized eggs, embryos and young tadpoles but shows a dramatic developmental surge in transcript levels peaking at metamorphic climax and returning to negligible levels in the adult. They demonstrated that TRβ expression was stimulated by thyroid hormones and this stimulation required protein synthesis. Schneider & Galton (1991) have similarly shown that expression of the TRα gene is also responsive to thyroid hormone level in tadpole erythrocytes. This thyroid hormone responsiveness of thyroid receptors will result in the autoinduction of receptors during metamorphosis and has been reproduced in *Xenopus laevis* cell culture as well as in tadpoles (Machuca & Tata, 1992). In both whole tadpoles and the cultured cells, TRα mRNA was present in the absence of thyroid hormones whilst negligible TRβ mRNA was measured. The degree of stimulation by thyroid hormones was greater for TRβ mRNA than for TRα mRNA. The autoinduction of TRs is sensitive to inhibition of protein synthesis (Machuca & Tata, 1992). Such autoinduction during metamorphosis is likely to be due to the fact that the TR gene has a TRE in its own promoter region. This may be especially true for the TRβ gene which, in *Xenopus laevis*, has been shown to have a number of TREs in its own promoter region (see Tata, 1996).

In erythrocytes from the chick embryo, there is dramatic decline in nuclear thyroid receptors during development (Dasmahapatra, Thomas & Frieden, 1987). This is probably due to changes in TRα, as TRα mRNA but not TRβ mRNA has been identified in chick erythrocytes (Forrest, Sjoberg & Vennstrom, 1990).

Measurement of receptor binding has shown that TRs are present in rat fetal tissues prior to the onset of fetal thyroid function. Brain receptor number increased threefold from day 14 to day 17, after which it remained constant until after birth when it rapidly increased to approximately adult levels by postnatal day 6. In contrast to this pattern, receptor numbers in liver and heart increased progressively throughout fetal and postnatal periods towards the level found in adult rat tissues (Perez-Castillo *et al.*, 1985). The postnatal rise in receptor number in rat liver is almost completely due to increases in TRβ which is absent in the fetal rat liver but responsible for approximately 80% of total binding in the liver of the young adult rat. TRα binding remains relatively constant during this period and thus declines from being responsible for 100% of liver T3 binding prior to birth to approximately 20% in the adult rat (Rodd *et al.*, 1992). Although there are dramatic changes in mRNA for TRs during this period, T3 binding capacity remains little changed (Strait *et al.*, 1990).

(2) Effects of thyroid hormones on vertebrate development

Thyroid hormones have effects on several tissues during development, the most notable being the central nervous system. That their deficiency during development permanently impairs brain function in the adult has been known for some time and has been reviewed by a number of authors (e.g. Legrand, 1986; Morreale de Escobar *et al.*, 1987; Nunez *et al.*, 1991; Porterfield & Hendrich, 1993; Oppenheimer & Schwartz, 1997). In humans, there are three clinical situations where such effects are manifest. They are, in order of decreasing severity of brain damage, (a) substantial endemic goitre associated with environmental iodine deficiency, (b) congenital hypothyroidism in the newborn and (c) maternal hypothyroxinemia during gestation. Such maldevelopment is characterized by deaf-mutism, mental retardation and motor disorders and can be remedied by hormone replacement initiated soon after birth. If hormonal replacement is delayed, the effects on the central nervous system can be irreversible. As well as thyroid deficiency impairing development, excess thyroid hormone levels are also detrimental to normal brain development (as they are also for amphibian metamorphosis). Most of our knowledge concerning the effects of thyroid hormones on brain development concerns the laboratory rat, in which the important periods are both the fetal and (particularly) the neonatal period. It was long thought that maternal thyroid hormones did not cross the placenta of eutherian mammals and were thus not important. However, the extensive studies of Morreale de Escobar and Escobar del Rey and

others have conclusively shown that thyroid hormones of maternal origin are found in the early fetus before onset of fetal thyroid function and their absence during early fetal life can have developmental effects (see Morreale de Escobar *et al.*, 1985, 1987). Maternal hypothyroidism during the first half of gestation has been shown to compromise the normal development of rats during the second half of their gestation (Bonet & Herrera, 1991).

The rat brain at birth is at the same stage as the human brain at 5–6 months of gestation and consequently, stages of brain development that take place during the last trimester of human development occur postnatally in the rat. This period also corresponds to the time of the developmental surge of thyroid hormones in the rat. Recently, the culturing of fetal and neonatal brain cells has given substantial insight into the cellular mechanisms involved, but much still remains unknown.

The early work of Eayrs and Legrand in the 1960s demonstrated that thyroid deficiency results in altered brain anatomy and dramatically decreased neuronal connectivity. This is largely due to a drastic decrease in axonal and dendritic outgrowth of neurones and synaptogenesis. In the brain, thyroid hormones not only influence the development of neurones but also that of glial cells. For example, thyroid hormone deficiency results in reduced myelination of neuronal axons.

When rats are thyroidectomized at postnatal day 10, there results a decrease in the density and a changed distribution of spines from the apical shaft of pyramidal neurons in their visual cortex, which is a manifestation of reduced neuronal connectivity. If T4 replacement is initiated at postnatal day 12, then spine density and distribution are similar to the control (euthyroid) condition. If however, T4 replacement is not commenced until postnatal day 30, spine density and distribution remains identical to that observed in the thyroidectomised group (Morreale de Escobar *et al.*, 1987).

Possibly the most dramatic visual manifestation of reduced arborization during hypothyroidism is observed in the characteristic dendritic tree of Purkinje cells in the cerebellum (see Legrand, 1986, p. 518). Growing axons and dendrites must reach their synaptic partners at an appropriate time and the correct spatio-temporal timing of neurite outgrowth is an essential component of the development of normal neuronal networks. Hypothyroidism retards the rate of growth of neurite outgrowths and thus the rate of migration of neurones towards their synaptic targets. The impaired growth and arborization of axons and dendrites in hypothyroid rats has been related to deficiencies of the cytoskeleton in peripheral and central neurons. The neuronal cytoskeleton includes microtubules and microfilaments. Microtubules are built from dimers of tubulin and also contain microtubule-associated proteins (known as MAPs) whilst microfilaments are composed of actin subunits. Actin is particularly important in axonal growth cones. Normal neurite growth depends both on the synthesis of cytoskeletal proteins and on their axonal transport. Axonal transport of tubulin has been measured in the optic nerve of the hypothyroid *hyt*/*hyt* mouse and found to be significantly slower than in euthyroid *hyt*/+ littermates (Stein *et al.*, 1991).

Tubulin barely self-assembles into microtubules at physiological concentrations and two protein families, MAP2 and Tau, act as promoters of tubulin assembly to form microtubules. Many of these proteins have a number of isoforms that are expressed at different developmental times. The mRNA levels for many of these proteins change in diverse ways during the period of neuronal differentiation and maturation (e.g. Figueiredo *et al.*, 1993). It has been suggested that thyroid hormones are biphasic (i.e. stimulating early, whilst inhibiting later) in their influence on cytoskeletal proteins and their respective mRNAs (Biswas, Pal & Sarkar, 1997). However, a better description of these findings is that thyroid hormone deficiency delays the normal developmental program of expression of these cytoskeletal components. Whilst the mRNA levels for $\alpha 1$ tubulin in cerebellum are elevated in hypothyroid rats compared to euthyroid control rats during the first 3–4 postnatal weeks (Aniello *et al.*, 1991; Figueiredo *et al.*, 1993), $\beta 4$ tubulin mRNA levels show the opposite effect, being reduced in hypothyroid compared to euthyroid rats (Aniello *et al.*, 1991). Both effects can be interpreted as hypothyroidism delaying the normal expression of tubulin isoforms. In a similar manner, thyroid hormone deficiency delays the transition between immature and mature MAP2 and Tau variants. In this case, the influence of thyroid hormones appears to be at the level of the splicing mechanism, that is, post-transcriptional (Nunez *et al.*, 1991).

The developmental profile for β-actin mRNA from normal (euthyroid) rat cerebra shows a peak at postnatal day 5 whilst in hypothyroid rats the peak occurs at postnatal day 10 (Poddar *et al.*, 1996). *In vitro* transcription measurements demonstrate a depressed actin gene transcription in hypothyroid cerebra that can be remedied by incubation of nuclei

with T3. By contrast, *in vitro* tubulin gene transcription rates are similar in hypothyroid and euthyroid cerebra and incubation of nuclei with T3 has no effect on tubulin gene transcription. This suggests that whereas transcription represents an important level of thyroid hormone control of the actin gene, thyroidal influence over tubulin expression is primarily post-transcriptional (Poddar *et al*., 1996). Levels of actin and its mRNA have also been measured in cultures of fetal human brain during the second trimester of gestation and it has been demonstrated that there is a peak in actin mRNA levels at week 18 of gestation, which corresponds with both a significant increase in cytoskeletal actin levels as well as the period when thyroid hormones in the culture medium stimulate actin production (Pal, Biswas & Sarkar, 1997). Thyroid hormones affect cytoskeletal actin and its mRNA during the maturation, in culture, of astrocytes obtained from neonatal rat brain and also have a distinctive influence on the external morphology of such astrocytes (Paul *et al*., 1996).

Neural networks are formed by neurite growth cones migrating along predictable routes to form synapses with specific target cells. The pathways of this migration are determined by proteins of the extracellular matrix, especially laminin, which is essential for synapse formation and cell survival as well as neurite migration. In the brain, astrocytes participate in the formation of these migration pathways by synthesizing and secreting laminin, which is a major component of the extracellular matrix of the developing brain. Laminin is bound to the astrocyte cell surface by specific transmembrane receptors known as integrins, which in turn are held in specific locations on the astrocyte surface by the actin microfilament network in the cytoplasm of the astrocyte (Reichardt & Tomaselli, 1991). In a potentially very important paper, Farwell *et al*. (1995) have shown that T4-treated astrocytes in culture readily attach to laminin whereas attachment was delayed for thyroid-hormone-deficient astrocytes. The T4-dependent laminin-integrin interactions provide a mechanism whereby T4 might influence neuronal migration and development. This effect has similarities to the previously described effects of thyroid status on the D2 deiodinase in astrocytes where specific T4-enzyme-actin interactions are necessary for the rapid internalization of the D2 deiodinase (Farwell & Leonard, 1992). In both cases, T4 and rT3 are considerably more effective than T3.

It is tempting to speculate that both the continual presence of T4 and the high levels of rT3 present (largely due to the predominance of inner ring over outer ring deiodination) during early development has an important functional role in facilitating interaction between body cells and the extracellular matrix. Together with the recent confirmation that astrocytes have an important support role for neurones and their function in the adult (see Magistretti & Pellerin, 1999) these findings make this an area very worthy of further investigation.

The expression of the different isoforms of nuclear thyroid receptors varies between different types of fetal brain cells. TRα-1 is found in both neuronal and astroglial cells, whilst TRβ-1 is localized to neuronal cells and the non-hormone-binding TRα-2 is restricted to astrocytes (Leonard *et al*., 1994). It has recently also been demonstrated that D2 deiodinase is primarily expressed in glial cells, rather than neurones, in the neonatal rat brain (Guadano-Ferraz *et al*., 1997).

The regions of the brain that mature late are those most severely affected by hypothyroidism. This includes the cerebellum, where the external germinal layer persists and remains the site of mitosis for an extended period in hypothyroidism. In germinal cells, thyroid hormones affect cell maturation rather than cell replication (Legrand, 1986). Myelogenesis is also retarded in hypothyroidism. The retardation effect of hypothyroidism is also manifest in mRNA levels for four brain proteins during postnatal development of the rat (Strait, Zou & Oppenheimer, 1992). In this study, the mRNA levels for calbindin, myo-inositol-1,4,5-triphosphate (ICP_3) receptor, Purkinje cell protein-2 (PCP-2), and myelin basic protein (MBP) are all low at birth and show a similar postnatal profile in euthyroid rats. Hypothyroidism did not change the final level of these four mRNAs but did delay their increase, such that at postnatal day 15 the mRNA levels for all four brain proteins were much reduced in hypothyroid compared to euthyroid rats. Cotransfection experiments in this study suggested that the thyroid hormone effect on PCP-2 was mediated by the TRβ-1 isoform. Hypothyroidism had no effect on the mRNA levels for these four brain proteins after the first four postnatal weeks of development in the rat, and manipulation of thyroid status of late-fetal rats also had no effect on the mRNA levels for PCP-2 and MBP proteins, suggesting that these proteins are unresponsive to thyroid hormones prior to birth in rats (Schwartz, Ross & Oppenheimer, 1997). Two thyroid response elements have been identified for the PCP-2 gene, one in the 5′ upstream region (A1)

and one in the first intron (B1) and it has been proposed that the function of the A1 TRE is suppressed by a neighbouring silencer element operating after the initial period of thyroid hormone responsiveness (Zou *et al*., 1994; Anderson *et al*., 1997). A TRE has also been characterized for MBP (Farsetti *et al*., 1992) but examination of the 1100 base pair region upstream of the calbindin gene has failed to find a TRE (see Oppenheimer & Schwartz, 1997). The situation has been complicated by the recent finding that a knockout strain of mice, devoid of TRβ-1, which was initially proposed to mediate the thyroid hormone effect, have a postnatal profile for PCP-2 mRNA and MBP mRNA that is identical to that in normal mice that possess a TRβ (Sandhofer *et al*., 1998). These results indicate that either it is the TRα isoform that mediates this developmental effect, or that nuclear receptors are not involved. They also suggest that translation of the findings of *in vitro* transfection experiments to the *in vivo* situation should be made with caution.

Hypothyroidism during development also commonly results in deafness. Postnatal rats made hypothyroid from late-fetal stages show no auditory evoked potentials and also show no postrotatory nystagmus which demonstrates that vestibular function is also impaired in hypothyroidism (Meza, Acuna & Escobar, 1996). Little is known of the precise cellular targets of thyroid hormone effects on the auditory system. Effects are manifest both in the sensory organ itself as well as in the auditory cortex. In normal rats, cochlear potentials following auditory stimulation have been recorded from postnatal day 9 but in hypothyroidism no cochlear potentials could be elicited during the first 30 postnatal days of life (Uziel, Rabie & Marot, 1980). Hypothyroidism results in abnormal synaptogenesis of efferent boutons at the level of the cochlear outer hair cells (Uziel *et al*., 1983). In the brain itself, hypothyroidism results in a decreased number and abnormal distribution of apical shaft spines in pyramidal cells of the auditory cortex (Ruiz-Marcos *et al*., 1983). These changes were similar to those found in the visual cortex, by the same investigators, but were less responsive to thyroid hormone replacement and it was suggested that the auditory cortex pyramids were either more sensitive to a minor degree of hypothyroidism, or have a shorter critical period during which hormone replacement can remedy damage, than other cerebral or cerebellar neurones and structures.

Early studies showed that Na^+,K^+-ATPase activity of the both the cerebra and cerebellum of the rat increased during the first postnatal month (Valcana & Timiras, 1969). Hypothyroidism delayed this increase in brain Na^+,K^+-ATPase activity and the later thyroid hormone replacement was begun, the less was the remediation (Schmitt & McDonough, 1988). The effect of T3 on the mRNA levels of Na^+,K^+-ATPase subunit isoforms has been examined in a cell culture system of rat forebrain that reproduces *in vitro* the pattern of normal *in vivo* development. Although T3 influenced the levels of mRNA for some isoforms at a culture stage equivalent to postnatal day 7 it had no influence at a culture stage equivalent to birth. Transcription rates for each isoform differed markedly but remained stable for the 12 days of culture and were not affected by T3. The effects of T3 on mRNA levels for the Na^+,K^+-ATPase subunit isoforms are likely to be post-transcriptional (Corthesy-Theulaz *et al*., 1991).

Few studies on the developmental effects of thyroid hormones on the brain and its function have been carried out in non-mammalian vertebrates. One such study, however, suggests that the thyroid hormones play a role in olfactory learning and imprinting in salmonid fish during the parr-smolt transformation (Morin, Dodson & Dore, 1989).

Thyroid hormones influence the development of tissues other than brain. For example, in muscle, embryonic myosin isoforms are replaced by neonatal isoforms which in turn are replaced by adult-type myosin heavy chain isoforms during early development. In rats, the postnatal surge in plasma T4 concentration coincides with the transition from neonatal to mature fast myosin isoforms in fast muscles and is retarded by hypothyroidism and precociously induced in hyperthyroidism (Gambke *et al*., 1983; Butler-Browne, Herlicoviez & Whalen, 1984). The times of such transitions vary between muscles and hypothyroidism delays, whilst hyperthyroidism accelerates, such transitions (d'Albis *et al*., 1990). Similarly, in humans, hyperthyroidism has been shown to result in precocious accumulation of adult myosin heavy chains early in postnatal development (Butler-Browne, Barbet & Thornell, 1990). Although hypothyroidism retards developmental changes in myosin expression in chick embryo skeletal muscles (Gardahaut *et al*., 1992), replacement of neonatal myosin isoforms with adult isoforms is not influenced by thyroid hormones in the turkey (Maruyama, Kanemaki & May, 1995). In amphibians, a moderate increase in thyroid hormone levels is sufficient to induce differentiation of adult fibre types and the production of adult myosin isoforms in skeletal muscle (Chanoine *et al*., 1987). In

a salmonid fish, the Arctic charr *Salvelinus alpinus*, T3 accelerates the neonatal-adult isoform transition to a small extent, but is not as effective as increased temperature (Martinez *et al.*, 1995). In the metamorphosing flounder, T4 exposure accelerates muscle developmental changes whilst exposure to thyroid inhibitors delays such changes (Yamano *et al.*, 1991).

During postnatal muscle development in the rat, a dramatic increase in the ability of the sarcoplasmic reticulum to transport Ca^{2+} has been observed. This increase is not observed in hypothyroid rats but can be restored by T3 (Simonides & van Hardeveld, 1989). Interestingly, this developmental response of muscle is unlike brain, in that it does not appear to have a critical period during which thyroid hormone replacement is necessary to restore normal development. Hypothyroid rats given replacement T3 at postnatal week 6 show the same response as those given replacement T3 in the first postnatal week (Simonides & van Hardeveld, 1989).

Mild hypothyroidism has also been shown to influence muscle development in the postnatal pig, in which it resulted in reductions in activities of both Na^+,K^+-ATPase and Ca^{2+}-ATPase in several muscles but only limited effects on the fibre composition of skeletal muscles (Harrison *et al.*, 1996). Developmental regulation of cation pumps in skeletal and cardiac muscle has been reviewed by Dauncey & Harrison (1996). In the rat, there are postnatal changes in the abundance of mRNAs for different subunit isoforms for Na^+,K^+-ATPase during the first postnatal weeks that differ from the profile in the brain and other tissues (Orlowski & Lingrel, 1988) but it is not known if these are influenced by the postnatal T4 surge.

The importance of the developmental thyroid hormone surge for muscle maturation has also been shown in the precocial sheep where this T4 surge occurs prenatally. Thyroidectomy *in utero* resulted in slower axon conduction in motor neurones (possibly due to thyroid hormone effects on myelination), and slower and weaker contractions of fast muscle, but had lesser effects on slow muscles (Finkelstein *et al.*, 1991). Such thyroid hormone effects on isolated muscle function are likely to be combinations of effects on myosin composition as well as sarcoplasmic Ca^{2+} and sarcolemmal Na^+/K^+ dynamics.

In the heart of the newborn rat, the sarcoplasmic reticulum is poorly developed and the cardiac cycle is more dependent on Ca^{2+}fluxes across the sarcolemma than across the sarcoplasmic reticulum membrane. During the first postnatal weeks the importance of the sarcolemmal Na^+/Ca^{2+} exchanger diminishes and the role of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2) increases. Inhibition of thyroid function during this time results in a lower level of both the SERCA2 protein and its mRNA and a higher level of the sarcolemmal Na^+/Ca^{2+} exchanger and its mRNA, with even greater effects on the respective transport activities of these two proteins (Cernohorsky *et al.*, 1998). Administration of T3 during this period had no effect on the levels of either protein and their respective mRNAs, however, it did result in an increase in the Ca^{2+} transport activity of SERCA2 and a decrease in that of the sarcolemmal Na^+/Ca^{2+} exchanger. The additional effect on the activity of SERCA2 is suggested to be due to either changes in phospholamban or membrane lipids (or both), whilst the effect on the activity of the Na^+/Ca^{2+} exchanger is suggested to be due to effects of altered Na^+,K^+-ATPase activity or membrane lipid changes (or both). Thus, the thyroid hormones are necessary for the reciprocal changes in expression of the cardiac sarcolemmal Na^+/Ca^{2+} exchanger and SERCA2 during normal postnatal development and also have influences on their respective activities that are independent of changes in the expression of their respective genes (Cernohorsky *et al.*, 1998).

It has been shown that T3 is a potent stimulant for embryonic myoblasts to differentiate in culture. A transient rise in cyclic AMP levels appears essential for the terminal differentiation of such myoblasts and T3 causes both an earlier and a greater cyclic AMP concentration increase (Marchal *et al.*, 1995).

The influence of the thyroid hormones on cartilage and bone has been recently reviewed by Williams *et al.* (1998). When various bones and cartilage are removed from one-day-old neonatal rats and transplanted under the kidney capsule of euthyroid and hypothyroid rats it was shown that the growth and differentiation of endochondral bones was more dependent on thyroid hormone levels than was that of membranous bones and cartilage. The former develop their thyroid hormone dependence before postnatal day 4 whilst the latter develop such dependence at the end of the first postnatal week (Liu & Nicoll, 1986).

The transfer of immunoglobulins across the intestinal mucosa is a process that diminishes during normal postnatal maturation of the mammalian intestine. In two-week-old rats, T4 administration stimulated structural and functional maturation resulting in a decreased fluidity of the microvillus membrane and diminishing uptake of immuno-

globulin (Israel *et al.*, 1987). In the rat, there are significant changes in the activities of a number of intestinal enzymes in the third postnatal week. Some enzymes decrease (e.g. lactase and acid β-galactosidase), whilst others increase in activity (e.g. sucrase and glucoamylase). As well as plasma T4 concentration, plasma corticosterone concentration also increases during the second postnatal week and is an important factor in this postnatal maturation of intestinal enzymes. Treatment with T4 increases serum corticosterone levels during this period and also synergistically heightens the effects of exogenous glucocorticoid on intestinal enzyme maturation although it does not in itself enhance maturation of intestinal enzymes (McDonald & Henning, 1992). That the thyroid is generally involved in the normal maturation of the vertebrate digestive system is illustrated by the finding that exposure to T4 stimulates precocious development of the gastric gland in premetamorphic flounder whilst inhibition of thyroid function inhibits gastric development during flounder metamorphosis (Miwa, Yamano & Inui, 1992).

An interaction between corticosterone and thyroid hormones has also been observed with respect to amphibian metamorphosis and has recently been reviewed by Hayes (1997). Exogenous corticoids in early development inhibit metamorphosis whilst in late development they accelerate metamorphosis. They interact with the thyroid axis in multiple ways. For example, in erythrocytes from premetamorphic tadpoles, the normal thyroid hormone stimulation of TRα number is inhibited by corticosterone (Schneider & Galton, 1995), whilst it has been shown that corticosterone stimulates 5′-deiodination but inhibits 5-deiodination in premetamorphic tadpoles resulting in an increased conversion of T4 to T3 and thus an increase in body T3 levels (Galton, 1990). Corticoids can also accelerate or inhibit tadpole hindlimb growth and development depending on the stage of spontaneous development (Wright *et al.*, 1994). Such hormonal interactions are the likely mediators of ecological and environmental effects on amphibian development. Cortisol and T3 also interact in larval development of a fish species (Kim & Brown, 1997).

Tissue remodelling during amphibian metamorphosis can be extensive. Metamorphic changes in the amphibian intestine involve the programmed cell death (apoptosis) of tadpole epithelium and its replacement by newly formed adult epithelium. Of several genes whose expression is increased early during amphibian metamorphosis, some are transcription factors, some are involved in metabolism whilst others are involved with extracellular signalling (Shi, 1996). The role of two of these latter genes has recently been discussed (Stolow *et al.*, 1997) and these authors have stressed the important role of the extracellular matrix and the transfer of information between different cell types during development. For example, the development of the adult epithelium requires the presence of larval connective tissue (Ishizuya-Oka & Shimozawa, 1992) and without it, the only observed morphological change in cultures of intestine treated with thyroid hormones is apoptosis of the larval epithelium. The proliferation and differentiation of the adult epithelium is absolutely dependent on the presence of larval connective tissue. This may present an interesting example for other cases of developmental changes. If cell to cell information transfer is an important component of developmental changes then thyroid hormone effects that enhance such transfer may speed up development; inhibition of such information transfer would be likely to retard developmental change. This means that thyroid hormone effects need not be genomic to influence development but could for instance act at a membrane level to speed up information transfer and consequently development. Interactions between cells and the extracellular matrix can result in transcriptional regulation of cellular genes (see Stolow *et al.*, 1997).

Apoptosis is also observed in other tissues (notably the tadpole tail) during amphibian metamorphosis (for review see Tata, 1994). Thyroid hormones play two distinctive roles during cell death in the tadpole tail. They induce and promote keratinization (programmed cell death with terminal differentiation) in epidermal cells of the tail and body, and influence proliferation of these two types of cells in opposite directions. T3 inhibits DNA synthesis in tail cells, but stimulates the same process in body cells (Nishikawa, Kaiho & Yoshizato, 1989). Whilst T3 accelerates keratinization of body cells, these cells will gradually keratinize *in vitro* without T3. Similarly, expression of the adult-type keratin gene in head skin during larval development of *Xenopus laevis* has been demonstrated to be activated by two steps: one independent and one dependent on T3 (Mathisen & Miller, 1987).

One of the changes that occur during amphibian metamorphosis is the development of the capacity for urea biosynthesis in the adult amphibian liver. A key enzyme for urea biosynthesis is carbamyl phosphate synthetase (CPS); CPS mRNA levels and

CPS enzyme activity are increased when premetamorphic tadpoles are exposed to T4 (Morris, 1987) and T3 (Galton *et al.*, 1991*a*). Thyroid hormones have the opposite effects in rat liver in that urea biosynthesis is stimulated by hypothyroidism (Marti *et al.*, 1988). Interestingly, whilst T4 stimulates synthesis of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in rat liver, it has the opposite effect in tadpole liver (Morris, 1987). Recently, it has been demonstrated that thyroid hormone induction of enzymes for urea synthesis (including CPS) during both spontaneous and induced metamorphosis of *Rana catesbeiana* is a secondary response due to a transcription factor in the amphibian liver that is homologous to the mammalian transcription factor C/EBPα. The mRNA for this transcription factor accumulates early in response to thyroid hormones, and the product of this mRNA can bind to and transactivate the promoters of CPS and ornithine transcarbamylase (another urea biosynthesis enzyme) in the liver of *Rana catesbeiana* (Chen & Atkinson, 1997). These results suggest that there is a cascade of gene activation induced by thyroid hormones during amphibian metamorphosis. Intriguingly, examination of thyroid hormone influence on the levels of the C/EBPα and C/EBPβ proteins, as well as their respective mRNAs, in the liver of the rat during early postnatal development suggests that the influence of thyroid hormones on these transcription factors is either translational or post-translational (Menendez-Hurtado *et al.*, 1997). Stimulation of malic enzyme in cultured chick embryo hepatocytes by T3 has also been suggested to occur primarily at a post-transcriptional level (Back *et al.*, 1986).

Many developmental changes that have been examined appear to be associated with the developmental surges of T4 and T3 levels. My impressions from the literature concerning thyroid hormone influences on development is that when single time points in development are investigated the conclusions are that thyroid hormones act, in some cases, to increase and in others to decrease the expression of genes, sometimes even the same genes at different times. However, when multiple time points are examined, a more consistent pattern emerges. This is that exposure to exogenous thyroid hormones generally accelerates development, whilst inhibition of thyroid function retards developmental change. The acceleration of normal development with increased levels of thyroid hormone and retardation with thyroid hormone deficiency is, in a way, analogous to their effects in adult vertebrates where they predominantly speed up normal processes when present in excess, and mostly slow down normal processes when not present in adequate amounts.

The influence of thyroid hormones on vertebrate development is likely to be *via* several different modes of action. Many effects are likely to be direct genomic effects, whilst several others are probably non-genomic. Differentiating between such modes will involve several criteria. The observation that thyroid hormone exposure results in elevated mRNA levels for a particular gene is, by itself, not adequate evidence for a direct genomic mode of action. Direct genomic effects will be mediated by nuclear receptors binding to TREs in the genome and thus a TRE is a mandatory requirement for a direct genomic action. The role of thyroid effects on the membrane bilayers and changes in the rate of information transfer on development has not been investigated. This is presumably because of the widely held belief that thyroid hormones only act *via* the nuclear receptor mode.

Membrane lipid compositional changes during development of vertebrates are known but have been little researched. For example, Hoch (1988) reports an increase in the unsaturation index of lipids (mainly due to an increased PUFA content) in the liver of *Rana catesbeiana* during metamorphosis that is coincident with the T4 surge. Changes in membrane acyl composition, cholesterol:phospholipid ratio and membrane fluidity of various membranes have been observed during postnatal development of the rat (e.g. Hubner *et al.*, 1988; Rovinski & Hosein, 1983; Kameyama *et al.*, 1987) and the rabbit (e.g. Nagatomo, Sasaki & Konishi, 1984; Ricardo *et al.*, 1986; Schwartz, Lambert & Medow, 1992) as well as during embryonic development of the chicken (e.g. Schjeide, 1988; Fuhrmann & Sallmann, 1996; Rivas *et al.*, 1996). For example, in the rat there is a postnatal peak in the unsaturation of liver mitochondrial phospholipids (Pollack & Harsas, 1981) that is consistent with the developmental T4 surge. Similarly, there is a postnatal peak in the 20:4 content of plasma membrane phospholipids, as well as microsomal and mitochondrial phospholipids isolated from chick heart (Kutchai *et al.*, 1978). However, the role of thyroid hormones in these changes in membrane bilayer composition and their functional consequences are not known.

One of the most interesting recent findings is thyroid hormone enhancement of the laminin-integrin-actin interaction observed in astrocytes (Farwell *et al.*, 1995; Leonard & Farwell, 1997).

Whether this phenomenon is also applicable to other cells and their interaction with the extracellular matrix, and whether thyroid-hormone-membrane interactions have a role in this effect is, in my opinion, one of the most fascinating and important questions regarding the developmental effects of thyroid hormones yet to be answered. In this effect, T4 is much more potent than T3, and rT3 is nearly as potent as T4. In view of the importance of cell migration during development, this thyroid hormone effect may be related to a number of findings in vertebrates: (i) the presence of T4 in the eggs and embryos of vertebrates well before the development and maturation of thyroid gland function in the individual, (ii) the developmental surge of T4, and (iii) the preponderance of inner ring 5-deiodination early in development and the switch over to outer ring 5′-deiodination, with the consequence of high levels of rT3 early in vertebrate development and its decrease and replacement by T3 later at approximately the time of the developmental T4 surge.

V. SOME PERSPECTIVES

(1) Analogues as a source of knowledge

As well as natural analogues of T4 (i.e. T3, rT3, the three T2s, the two T1s, and thyronine and other products of T4 metabolism) a substantial number of other analogues have been synthesised and examined for their thyroid-hormone-like potencies. A substantial amount of this work has been carried out by Jorgensen and his colleagues and an excellent review of these findings is provided by Jorgensen (1981). For additional reviews, see Cody (1996) and Craik, Duggan & Munro (1996). Coupled with the study of the conformation of analogues, these studies have resulted in considerable insight into: (i) important aspects of the molecular interactions during the binding of thyroid hormones to various proteins, (ii) the relative importance of the various parts of the thyroid hormone molecules in their physiological effects, and (iii) the mode of action for various effects of thyroid hormones.

These analogues bind with different affinities to a variety of proteins, which include both the extracellular distributor proteins and intracellular proteins (notably nuclear thyroid receptors). Although binding affinities vary between vertebrate species, detailed measurement has generally been restricted to the human or rat proteins. Such relative binding affinities are presented in Table 4.

Binding to ALB is weak and insensitive to the stereochemistry of the alanine side chain with the major binding feature being the o-diiodophenolic structure in which the phenolic −OH group is ionized. Binding to TTR is highly sensitive to the stereochemistry of the alanine side chain with binding being primarily to the carboxylate ion of the side chain with the ammonium group weakening such binding. The o-diiodophenolic structure is also important for binding to TTR with the 3′ and 5′ iodine atoms being very important, together with a hydrogen bond formed between the ionized form of the phenolic −OH group and TTR N-terminal residues (which reside at the entrance to the TTR binding channel). In this light, it is of interest that the N-termini are longer and more hydrophobic in avian TTR compared to rat, sheep and human TTR, and that avian TTRs have a greater affinity for T3 than the mammalian TTRs (Chang *et al.*, 1998). Thyroid hormones bind to TBG with great affinity because this protein associates with both the carboxylate and the ammonium groups of the alanine side chain. As with TTR, the 4′ −OH in its ionized form together with the 3′ and 5′ iodine atoms are important in the binding of thyroid hormones to TBG (Jorgensen, 1981).

The *in vitro* binding of thyroid hormones and analogues to thyroid nuclear receptors (TRs) has been examined for intact rat liver nuclei and its solubilized receptor. The two preparations closely agree and both binding affinities are shown in Table 4. Whereas T3 binds to the plasma proteins with much less affinity than T4 (TBG, TTR and ALB have affinities for T3 that are respectively 9, 5 and 55 % of their affinity for T4), the opposite is the case with binding to thyroid nuclear receptors where the affinity for T3 is approximately 800 % of that for T4. TRs have less affinity for the D-isomer of the alanine side chain than the L-form. Binding is to the carboxylate ion of the alanine side chain, with the presence of an amino group reducing binding. Two aromatic rings separated by either an O, S or a C (with an inter-ring bond angle of approximately 120°) is also important. Non-polar groups on the 3- and 5- positions on the inner ring are important for binding to TRs. The greatest affinities are for iodine atoms in these positions, and their replacement with smaller groups (e.g. Br atoms or $-CH_3$ groups) gives lower affinities. The role of these inner ring iodine atoms appears to be to hinder rotation around the ether linkage and thus restrain the two aromatic rings such that they are perpendicular to each other. This role can be easily observed in the molecular

Table 4. *The relative potencies of iodothyronines in binding to selected proteins and exerting selected thyroid hormone effects*

Iodothyronine	L-T4	D-T4	L-T3	D-T3	rT3	3,5-T2	3′,3-T2	3′,5′-T2	3-T1	3′-T1	T	Reference
Relative binding affinity												
TBG (human serum)	1100	600	100		422	1	14	1		0	0	Jorgensen (1981)
TTR (human serum)	2100	56	100	4	167	2	15	48	0	1	0	Jorgensen (1981)
ALB (human serum)	180	182	100		182	55		155		27	0	Jorgensen (1981)
Intact nuclei (rat liver)	13		100		0	0	1					Jorgensen (1981)
Soluble TR (rat liver)	14		100		0	0	1					Jorgensen (1981)
Thyroid hormone effect												
Antigoitre assay (rat)	18	3	100	7	0	1	0	0		0		Jorgensen (1981)
Body growth (rat)	100		100									Evans *et al.* (1964)[1]
BMR (rat)	70	12	100			~ 100	~ 100					Pitt-Rivers & Tata (1959), Lanni *et al.* (1996), Moreno *et al.* (1997)
Isolated liver O_2 consumption (rat)	96		100		0	108	0	0	18			Horst *et al.* (1989)
Muscle *in vivo* sugar uptake (rat)	~ 70	~ 40	100	~ 70								Segal (1989)[2]
Thymocyte amino acid uptake *in vitro* (rat)	34	13	100	20	48	15	103	5	28	3	0	Goldfine *et al.* (1976)
Erythrocyte Ca^{2+}-ATPase *in vitro* (rat)	132	0	100	0	0	100						Davis *et al.* (1989)[3]
Brain D2 deiodinase *in vivo* (rat)	~ 1200		100		400							Silva & Leonard (1985)[4]
Amphibian metamorphosis (by immersion)	32	16	100	35	1	13		0	0	0		Jorgensen (1981), Pitt-Rivers & Tata (1959)
Amphibian metamorphosis (by injection)	10	3	100	50								Jorgensen (1981)
Membrane fluidity *in vitro* (liposomes)	~ 60	~ 60	100	~ 100		~ 10						Farias *et al.* (1995)[5]
Membrane antioxidant *in vitro* (rat)	~ 180		100			~ 50					0	Cash *et al.*(1967)[6]

All potencies are expressed relative to L-T3 (= 100), with relative potencies measured as less than 0.5 shown as zero.
[1]Both T4 and T3 (at 0.1 μg day^{-1}) resulted in similar growth of several body organs as well as body mass and metabolic rate.
[2]Calculated as average values for diaphragm, atria and ventricles after 0.1 μg per 100 g mass injection.
[3]Similar relative potencies also reported for sarcoplasmic reticulum Ca^{2+}-ATPase by Warnick *et al.* (1993).
[4]Values for effect on cortex D2 deiodinase activity, similar relative potencies measured for pituitary D2 deiodinase activity (Silva & Leonard, 1985) and GH3 cells *in vitro* (St Germain, 1985).
[5]Calculated from relative change in membrane fluidity of dimyristoyl-phosphatidylcholine liposomes due to 50 μM hormone at 36 °C.
[6]Calculated from relative % inhibition of liver mitochondrial membrane lipids at 1 μM hormone concentration.
T, thyronine; TBG, thyroxine binding globulin; TTR, transthyretin; ALB, albumin; TR, thyroid nuclear receptor; BMR, basal metabolic rate.

models for T4, T3 and 3,5-T2 in Fig. 1 (compared to the model of rT3). On the phenolic ring, the 4′−OH group is also important (in the non-ionized form), and affinity is enhanced by a lipophilic halogen, alkyl, or aryl group adjacent to this 4′−OH group. Strongest binding is obtained when the 3′-position is occupied by either an iodine atom or the similar-sized isopropyl group.

One of the *in vivo* tests of the thyromimetic activity of thyroid hormone analogues is the rat goitre prevention test, which is a measure of the amount of analogue required to inhibit thyroid gland enlargement in rats in which thyroid gland function is inhibited and a goitre is developing. It is a measure of the potency of the analogue to inhibit TSH release from the anterior pituitary and is also given in Table 4. There is a strong correlation between the relative affinity of nuclear TRs for an analogue and its potency in the rat antigoitre assay (Dietrich *et al.*, 1977). Discrepancies between TR binding affinity for and the thyromimetic potency of particular analogues have been variously explained. For example, the discrepancy between the low TR affinity of analogues lacking the 4′−OH but their relatively greater thyromimetic potency has been explained by the *in vivo* 4′-hydroxylation of such analogues (Oppenheimer, 1983). The relatively low thyromimetic potency of Triac (the acetic acid analogue of T3) compared to the very high TR affinity for it, has been explained by its more rapid metabolism compared to T3 (Oppenheimer, 1983).

The goitre prevention assay tests only one of the myriad effects of thyroid hormones. Other assays of thyromimetic potency that have been used in the past are the stimulation of metabolic rate of rats and the stimulation of amphibian metamorphosis. An earlier compilation of analogue potency included these other two assays (Pitt-Rivers & Tata, 1959) and showed that the relative potency of some analogues varied between the types of assay (Fig. 12). As can be seen from Fig. 12, there are some large discrepancies among assays. For example, whilst T3 had a thyromimetic potency that was 400–500 % that of T4 in the goitre prevention and the metamorphosis assay, it was only 67 % more potent than T4 in the metabolic rate assay. Whilst 3,5,3′-tribromothyronine had 20–28 % of the T4 potency in goitre prevention and stimulation of metamorphosis it was 90 % as potent as T4 in stimulating metabolic rate. Conversely, 3,5-diiodo-3′,5′-dichlorothyronine was 22–48 % as potent as T4 in stimulating metamorphosis and preventing goitre but had almost no effect on metabolic rate being only 0.4 % as potent as T4. Alteration of the alanine side chain produced a series of analogues that were considerably more potent than T4 in stimulating metamorphosis but considerably less potent than T4 in preventing goitre or stimulating metabolic activity. Whilst some of the high relative potencies for stimulation of metamorphosis obtained for these later analogues were measured by *in vitro* exposure, and lesser relative potency values were obtained following *in vivo* injection into tadpoles (see Jorgensen, 1981), they still remained more potent than T4 in this thyromimetic property but less potent than T4 in the other assays. Whilst comparison of the stimulation of metamorphosis with the other two assays is complicated by the fact that different species are involved, this is not the case when comparing the other two assay systems, which were both measured in the rat. The finding that analogues have different relative potencies when different thyroid hormone effects are measured is evidence that the effects themselves probably involve different modes of action.

Whilst the nuclear TR has an affinity for T3 that is 800 % that for T4, T3 has a thyromimetic potency for stimulating growth and metabolism in rats that is similar to that for thyroxine (Table 4). This has been explained by assuming that T4 is converted to T3 to exert all its effects (see Oppenheimer, 1983). Another explanation is that thyroid hormone effects on metabolism and growth are not predominantly mediated by thyroid nuclear receptors. In contrast to their almost equivalent potency in stimulating metabolism and growth, T3 is approximately four-fold more potent than T4 in suppression of rat serum TSH levels and the non-halogenated analogue, 3,5-dimethyl-3′-isopropyl-thyronine has approximately 10 % of the potency of T4 (Chopra *et al.*, 1984). The relative potencies of these two analogues in the goitre prevention assay are similar to the relative TR affinities (Jorgensen, 1981). That the thyroid stimulation of metabolic activity is not predominantly mediated by nuclear receptors is also supported by the fact in humans that doses of Triac sufficient to suppress TSH secretion had no effect on metabolic rate whilst doses of T4 sufficient to suppress TSH secretion resulted in a significant stimulation of metabolic activity (Bracco *et al.*, 1993).

The relative potencies of iodothyronines in several other effects are also presented in Table 4. These relative potencies differ markedly from the relative binding affinity of nuclear receptors for the iodothyronines and thus imply that these effects are not mediated by nuclear receptors. Within this group

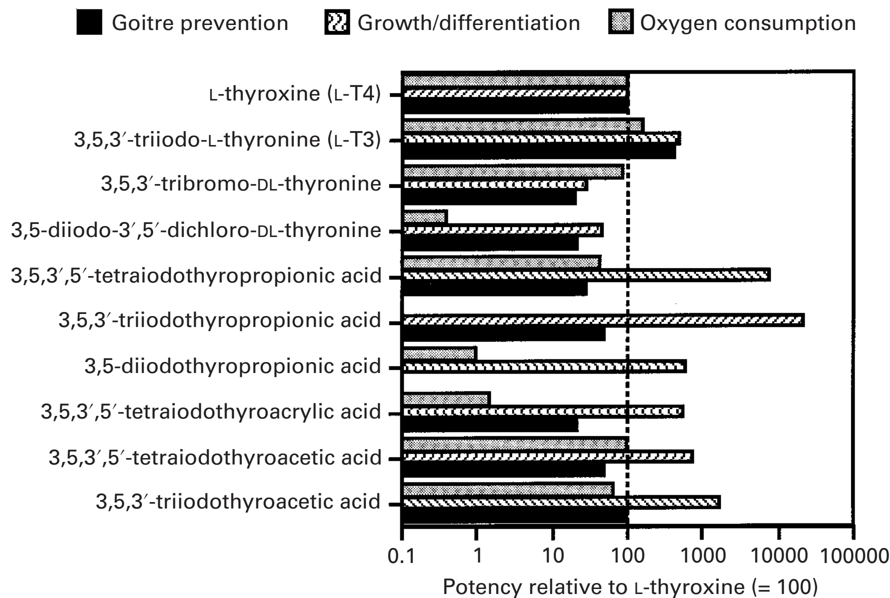

Fig. 12. A comparison of the potencies (relative to T4) of various thyroid hormone analogues on three different thyroid hormone effects. Data are from Pitt-Rivers & Tata (1959). The goitre prevention and oxygen consumption assays were performed in rats. The growth/differentiation assay involves induction of metamorphosis in amphibians.

there appear to be several different patterns. Metabolic rate and nutrient uptake may represent one group, Ca^{2+}-ATPase stimulation another, and the effects on D2 deiodinase still another. This latter effect appears to involve the same mechanism as the interaction of astrocytes with the extracellular matrix. T4 and rT3 may turn out to be more important in the early development of vertebrates than T3.

In Table 4, I have included two other effects of the thyroid hormones for which the relative potencies were obtained at high concentrations. These are the only data available for these effects and the relative potencies for the various iodothyronines may change when they are measured at lower, more physiologically relevant, concentrations.

I have argued elsewhere in this review that the effects due to 3,5-T2 may not be physiologically relevant because plasma T2 levels are considerably lower than those of T4 and T3. However, the finding that T2 is almost as active as T3 in exerting various thyroid hormone effects, yet TRs show negligible binding affinity for it, raises serious questions about the assumption that most thyroid hormone effects are mediated by a TR mode of action.

There are several other effects of thyroid hormones where the relative potency of analogues does not agree with their TR affinity. For example, TRs show negligible affinity for 3,5-dimethyl-3′-isopropyl-thyronine yet it is the most potent stimulator of muscle sarcoplasmic Ca^{2+}-ATPase activity, and although there are substantial differences between TR affinities for T4 and T3, these two iodothyronines have almost equal influence on the activity of this Ca^{2+}-ATPase. Whilst the acetic acid analogue of T3 has no significant influence on Ca^{2+}-ATPase activity, the nuclear TR has a very high affinity for this analogue (Warnick *et al.*, 1993). Such effects would not appear to be initiated *via* nuclear TRs.

(2) **The early sixties revisited**

It is the purpose of this section to use the wisdom of hindsight to re-examine the seminal studies of Tata *et al.* (1962, 1963), Tata (1963) and Tata & Widnell (1966). These detailed the time course of changes in a substantial number of variables following T3 injection into thyroidectomised rats. Our understanding of biological systems has increased greatly since this time. For example, the beginning of the modern understanding of fluid membranes and their importance can be dated to almost a decade later (Singer & Nicolson, 1972). Similarly, the often misleading but powerful concept of 'rate-limiting enzymes' permeated the understanding of the control of metabolism at the time. The more sophisticated understandings from 'metabolic con-

trol analysis' which now guide our thinking also had their birth about a decade later (see Fell, 1997).

Tata and colleagues conducted their experiments within a paradigm that suggested that the primary action of thyroid hormones was on mitochondria and that these hormones acted to uncouple ATP production from oxygen consumption and substrate utilization. They showed that when more physiologically relevant concentrations of thyroid hormones were used *in vivo* that the mitochondria from both rat liver and muscle exhibited no change in their P/O ratio. This was correctly interpreted to show that thyroid hormones did not result in uncoupled mitochondria. However, they also showed that thyroid hormones stimulated liver and muscle mitochondrial respiration when a phosphate acceptor system is absent and thus provided early evidence that thyroid hormones increased mitochondrial proton leak, an effect studied extensively by Brand and others and discussed in Section III.4.

Tata *et al.* (1963) also included the time course of thyroid hormone influences on a number of non-mitochondrial enzymes presented in a series of Tables and concluded: 'it appears from our results that enzymes or functions linked firmly to membranous subcellular structure are more markedly affected during the early phase of thyroid hormone action than activities not dependent on structural integrity' (Tata *et al.*, 1963, p. 426). Some of their results are given in graphical form in Fig. 13. In this form, it becomes obvious that the injection of T3 resulted in substantial stimulation of those enzymes associated with microsomal membranes but had no consistent or substantial influence on those enzymes present in the cytosol. They also showed that T3 increased the efficiency of amino acid incorporation into microsomally synthesised proteins in liver but that T3 resulted in no change in tissue RNA concentration.

The interpretation of the time course of thyroid hormone effects on RNA synthesis in rats (Tata & Widnell, 1966) further focussed attention on the nucleus as the site of thyroid hormone action. The most dramatic increases were in (i) the incorporation of ^{14}C-labelled orotic acid into nuclear RNA, (ii) an increase in [^{32}P]phosphate into ribosomal RNA, (iii) an increased incorporation of ^{14}C-labelled amino acids into microsomal protein. Other effects included increased RNA polymerase activity that peaked 30–45 h after T3 injection. The sequence of these responses (especially the first) were very important evidence favouring a nuclear site of action for the thyroid hormones. The manner of their measurement, however, does not exclude other interpretations including changes in membrane permeability for these labelled substrates. It is quite feasible that thyroid hormone changes in the uptake dynamics of orotic acid into liver nuclei would result in increased measured incorporation into nuclear RNA. Such an interpretation is supported by the fact that although there was a 300% increase in ^{14}C-labelled orotic acid incorporation into nuclear RNA there was no change in the amount of nuclear RNA following T3 injection (see Fig. 7 in Tata & Widnell, 1966). In addition, the results of their study showed that after *in vivo* T3 injection, ribonucleoprotein particles, or the mRNA attached to them, or both, were more firmly bound to microsomal membranes. Thus, the results from these experiments can also be interpreted to support a membrane site of action for the thyroid hormones.

(3) Knockouts from the nineties

One of the major advances made possible by the revolution in molecular biology is the capacity to interfere with specific genes. In particular, the ability to produce 'knockout mice' has provided a new experimental tool. Of relevance to the current review are the recently published findings for a number of knockout mice strains, in which the genes for either the TRα receptors, the TRβ receptors or the thyroid hormone binding protein, TTR, have been made non-functional.

The TRβ knockout mouse strain has a recessive gene for nonfunctional TRβs (Forrest *et al.*, 1996*c*). Heterozygous mice have functional TRβ receptors whilst homozygote recessives have neither functional TRβ-1 nor TRβ-2. There is no change in TRα expression. Use of behavioural tests that had previously demonstrated learning disabilities in the hypothyroid (*hyt*) mouse (Anthony, Adams & Stein, 1993) showed that there were no differences between mice possessing and mice lacking functional TRβs (Forrest *et al.*, 1996*b*). Histological and histochemical analysis of the central nervous system revealed no obvious abnormalities in brain anatomy, including the cerebellum and hippocampus and structures known to be thyroid hormone sensitive (Forrest *et al.*, 1996*b*). When homozygous mice were tested for their hearing ability, they were observed to have a permanent deficit in auditory function over a wide range of frequencies, and as the auditory-evoked brainstem response had a normal waveform, although greatly diminished, it was suggested that

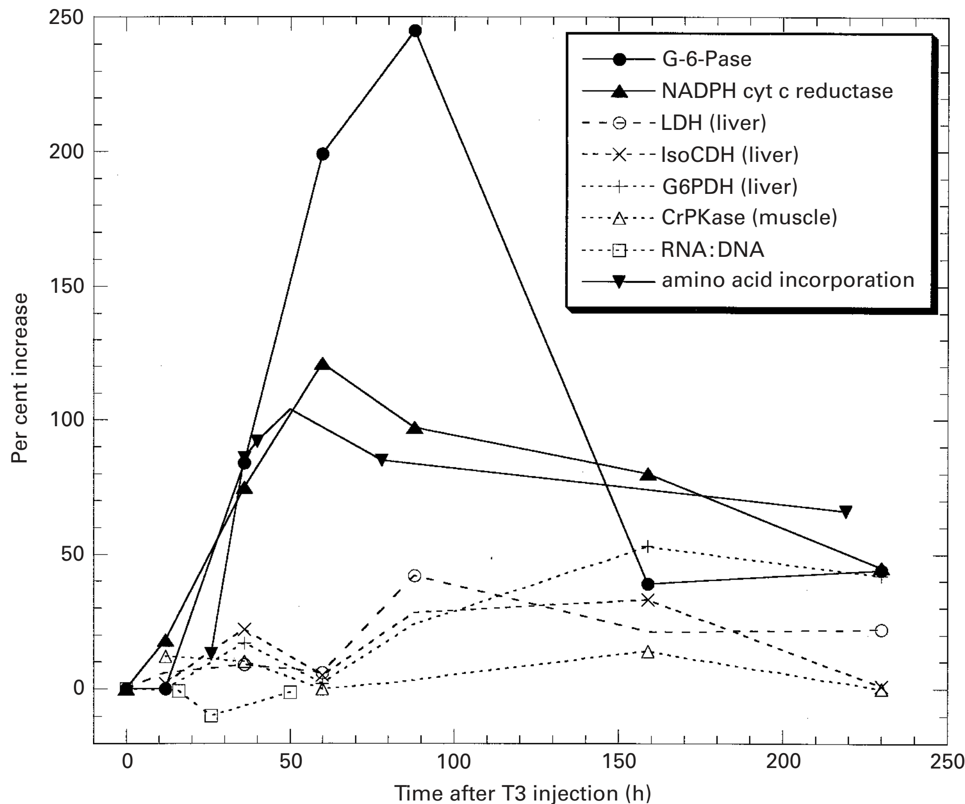

Fig. 13. Time course following T3 injection, of the activities of microsomal (solid symbols and unbroken lines) and cytosolic (open symbols and broken lines) enzymic activities and liver RNA:DNA (open square and unbroken lines) in the rat. Data are from Tata *et al.* (1963). G-6-Pase, glucose-6-phosphatase; NADPH cyt c reductase, NADPH cytochrome C reductase; LDH, lactate dehydrogenase; IsoCDH, Isocitrate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; CrPKase, creatine phosphokinase.

the primary deficit was at the cochlear level. They exhibited no other neurological defects (specifically no vestibular defects) and the developmental defect in hearing was not influenced by the maternal uterine environment (Forrest *et al.*, 1996*a*). Such TRβ-deficient mice appear to grow normally and become normally fertile adults. Litters were in normal Mendelian ratios which showed there was no differential prenatal mortality (Forrest *et al.*, 1996*b*). TRβ-null mice have a postnatal profile for PCP-2 mRNA and MBP mRNA that is identical to that of normal mice (Sandhofer *et al.*, 1998). Other than the hearing defect, the only other (so far) reported abnormality relates to the thyroid hormone regulation axis, especially TSH responsiveness.

Measurement of serum TSH levels in TRβ-null mice showed them to have 3–4 times the serum TSH concentration of mice with functional TRβs and similar differences were observed in serum T4 and T3 levels (both for total and free hormone levels). Elevated TSH is normally inconsistent with high serum thyroid hormone levels and similar to the 'resistance to thyroid hormone' syndrome observed in humans. These mice also had enlarged thyroid glands (Forrest *et al.*, 1966*c*), consistent with their high TSH levels. More recently, it has been shown that such TRβ-null mice respond normally to hypothyroidism (induced by dietary iodine deficiency) with an increased secretion of TSH but that they are deficient in their response to injection of thyroid hormones. Whereas mice with functional TRβ will completely suppress TSH secretion following thyroid hormone injection, TRβ-null mice are unable to suppress TSH levels below those observed in normal euthyroid mice (Weiss *et al.*, 1997). Thus, TRβ-null mice exist in a hyperthyroid condition.

Two different TRα knockout strains have been created. In one strain, only the TRα-1 isoform is nonfunctional (Wikstrom *et al.*, 1998), whilst in the

other strain both TRα–1 and the non-thyroid-hormone binding c-erbAα-2 are both nonfunctional (Fraichard *et al.*, 1997). I will refer to them here as TRα1-null and TRα-null mice, respectively.

The TRα1-null mouse is a homozygous recessive condition. Such mice are born in the expected Mendelian ratio suggesting no differential prenatal mortality. They are viable and survive to at least 18 months of age with both males and females being fertile and producing litters of a normal size. The animals appear healthy with no overt abnormalities detected at autopsy (Wikstrom *et al.*, 1998). No compensatory changes in TRβ expression are observed in TRα1-null mice. Such TRα1-null mice, at 2–4 months of age, show a reduction in serum TSH levels with no significant reduction in free T3 concentration but an approximately 30 % reduction in free T4 levels. They are thus slightly hypothyroid (Wikstrom *et al.*, 1998).

The TRα1-null mice also exhibited significant phenotypic variation in that they had heart rates that were nearly 20 % lower, and a body temperature that was on average 0.5 °C lower than wild-type controls (Wikstrom et al., 1998). However, whether this is due to the absence of TRα-1 or due to the hypothyroid state of these mice is unknown. That injections of T3 into TRα1-null mice resulted in an increase in both heart rate and body temperature illustrates that they were reponsive to thyroid hormone despite the absence of TRα-1, suggesting that the second explanation is most likely.

The TRα-null mouse is also a homozygous recessive condition. Young are born according to a normal Mendelian ratio suggesting no differential prenatal mortality. Similar to the TRα1-null mice, there was no compensatory change in TRβ expression. They exhibited no obvious external phenotype until postnatal week 2 when they ceased growing. At three weeks of age, although they had ceased growing, TRα-null mice appeared healthy and exhibited normal behaviour. Their internal tissues did not display any overt abnormalities and no cellular or morphological abnormalities could be detected. The cerebral cortex showed normal laminar organization, cytoarchitectonics and cortical parcellation with the organization of subcortical nuclei being normal (Fraichard *et al.*, 1997). However, at three weeks of age they appeared more severely hypothyroid than the TRα1-null mice. They had mRNA levels for βTSH that were approximately 70 % reduced compared to wild-type controls and serum levels of total T4, free T4 and total T3 that were 40 % lower than those of wild-type controls. They had very small thyroid glands in which some follicle cells exhibited vacuolar degeneration. By postnatal week 5 this hypothyroidism in the TRα-null mice had become extremely severe in that total T4 and total T3 levels were respectively 90 % and 60 % reduced compared to wild-type controls and serum free T4 concentration was below the limits of detection (Fraichard *et al.*, 1997). Although they had not exhibited any growth from postnatal day 10, from postnatal week 4 onwards TRα-null mice actually lost 30–50 % of their body mass and consequently died. Survival time varied with litter size and was probably influenced by lactational energy transfer from the mother.

Intriguingly, a very small number of TRα-null mice (approximately 1 %) spontaneously survived for up to 3–7 months. When TRα-null mice were given T3 for a short period at 3 weeks of age, a substantial number (40 %) survived for a further 3–6 months resuming a near-normal growth rate; when serum was measured several weeks after the T3 injections, they had the same levels of total T4 and T3 as wild-type control mice (Fraichard *et al.*, 1997). These findings together with those of Weiss *et al.* (1997), suggest that TRα-1, but not TRβ, is responsible for the up-regulation of TSH levels and that both TRα and TRβ are involved in the down-regulation of TSH levels, with TRβ being more potent than TRα (in that it is able to inhibit TSH secretion completely).

These results suggest the following scenario. Without both TRαs there is little likelihood that serum thyroid hormones will reach a level where TRβ alone can regulate their concentrations. If thyroid hormone levels can be elevated artificially to such a level, then TRβ alone can regulate thyroid hormone levels by negative feedback. The difference between TRα1-null mice and TRα-null mice suggests an important role for TRα2 in that it may stimulate TSH release irrespective of serum thyroid hormone levels. It may be an important component of the rising phase of the T4 surge during development. The role of TRH in this feedback system is presently unknown. These findings give some insight into the developmental T4 surge, which in normal mice occurs during these first postnatal weeks, and suggests that it may be an important process involved in the 'kicking in' of the regulatory system which then homeostatically regulates a relatively constant plasma level of thyroid hormones during adult life. It also suggests that the two different receptor isoforms may have different roles during this surge; the TRα isoforms being re-

sponsible for the rising phase whereas the TRβ isoform is more important in the falling phase of the surge.

Similarly, the TRα-null mice showed phenotypic effects. These were only manifest after the first postnatal week and included delayed maturation of the small intestine as well as delayed bone development. That these were not direct effects due to the absence of TRαs but were more likely to be due to the postnatal hypothyroidism is illustrated by the fact that TRα-null mice that were given T3 injections also had normal maturation of small intestine and bone following these injections (Fraichard *et al.*, 1997).

Differentiation between genomic and non-genomic modes of thyroid hormone action could be determined if mice in which both TRα and TRβ are nonfunctional were examined. Because of the undoubted role of both receptor isoforms in the hypothalamus-anterior-pituitary-thyroid axis, it would be necessary to maintain plasma T4 levels artificially (including its developmental surge) in such animals.

The TRβ-1 receptor has also been deleted in a GH3 cell culture system with the use of an antisense RNA vector directed against TRβ-1. Similar to the situation observed in knockout mice, there were no compensatory changes observed in TRα expression. However, within the TRβ isoforms, there were compensatory changes observed. In GH3 cells that had TRβ-1 isoform expression repressed, there was an increase in TRβ-2 expression and also an increase in basal expression, as well as the T3-stimulated expression of the growth hormone gene (Ball *et al.*, 1997). Such a finding suggests that within cells there are TR-isoform-specific expressions of thyroid hormones and that one TR isoform may not compensate for another.

Another knockout mouse of relevance to this review is the TTR-null mouse. This condition is also a homozygous recessive condition and such mice are born in the expected Mendelian ratio suggesting no differential influence on fetal development. TTR-null mice display no obvious postnatal phenotypic abnormalities as determined morphologically and by histopathological analysis. They exhibit the same longevity as wild-type siblings and both males and females have normal fertility. TTR was found neither in the plasma of such mice nor was it produced by the choroid plexus (Episkopou *et al.*, 1993). Although total plasma T4 concentration was reduced by 50% and total T3 levels were reduced by 15% in TTR-null mice compared to wild-type siblings, there was no difference in the plasma levels of either free T4 or free T3 nor in plasma TSH concentrations. Similarly, the activities of three thyroid-hormone-sensitive enzymes were unaffected in TTR-null mice (Palha *et al.*, 1994). When the transfer kinetics of tissue uptake of labelled T4 was examined in such mice it was observed that although the T4 clearance from plasma to brain was reduced by approximately 40%, and measured brain T4 content was reduced by approximately 30% in TTR-null mice, the T3 content of brain was not significantly different compared to wild-type siblings. Tissue T4 content was not significantly affected in liver and kidney (Palha *et al.*, 1997). Such results are indicative of redundancy of plasma proteins and emphasize the importance of both the 'free hormone' concept and the brain D2 deiodinase. TTR also normally transports plasma retinol complexed with plasma retinol-binding protein; the transport and metabolism of retinol in TTR-null mice is reviewed by Wolf (1995).

(4) Resistance to thyroid hormones

In 1964, a young deaf-mute girl was involved in a road accident in Los Angeles and when she was X-rayed in hospital, it was observed that all major secondary ossification centres in her bones were stippled. This observation together with a slight enlargement of her thyroid gland suggested neonatal hypothyroidism but her serum protein-bound iodine was unexpectedly high and found to be in the hyperthyroid range. A follow-up examination of her siblings and parents resulted in the first description of the condition, now known as 'resistance to thyroid hormones' or RTH (Refetoff, DeWind & DeGroot, 1967; Refetoff, 1994). There have been several excellent recent reviews of this condition (Usala, 1995; Refetoff, 1996; Chatterjee, 1997; Beck-Pecoz, Asteria & Mannavola, 1997). Since the original description, several hundred patients have been recognised and categorised into two types of RTH; generalized RTH (i.e. GRTH) in which the subjects have (i) elevated levels of free thyroid hormones, (ii) inappropriately normal serum TSH levels, and (iii) a mosaic of phenotypes but can generally be regarded as clinically euthyroid. This is the predominant form representing approximately 85–90% of described cases, with the other form known as pituitary RTH (i.e. PRTH) exhibiting (i) elevated free thyroid hormone levels, and (ii) inappropriately normal TSH, but (iii) showing signs and symptoms

of hyperthyroidism. The difference between these two conditions is not distinct.

The phenotype associated with RTH is extremely variable with the most common features being the presence of goitre (95 %), tachycardia (80 %), hyperkinetic behaviour (72 %), and emotional disturbance (65 %). Other less frequently diagnosed features include cardiac disease (30 %), learning disabilities and speech impediments (both in 28 % of cases), growth retardation (19 %), attention-deficit hyperactivity disorder (11 %) and hearing loss in 8 % of cases (Beck-Pecoz *et al.*, 1997). The majority of cases are inherited (80–90 %) and are related to a variety of mutations in the TRβ hormone-binding domain which generally occur in one of three specific 'hot-spot' regions. All of the mutated TRβs for which it has been measured show a diminished T3 binding affinity (Usala, 1995). Most patients are heterozygous having both a mutant and a normal TRβ but the mutant TRβ inhibits the activity of the normal TRβ as well as TRα. This condition is described as a 'dominant negative' effect. One individual has been recorded as homozygous for a mutant TRβ and exhibited the most severe form of the syndrome having a resting heart rate of 190 beats/min. He died from cardiogenic shock complicated by septicemia. Interestingly, the subjects originally described were homozygous for a complete deletion of both TRβ alleles (Beck-Pecoz *et al.*, 1997). TRβ-null mice exhibit both the deaf-mutism and the serum thyroid hormone and TSH profile typical of this form of RTH. No mutations have been recorded for TRα in RTH patients.

The variability of the phenotype in RTH may be related to the variety of mutations in TRβ but individuals with the same mutation have been classified differently and even within families with the same mutation, phenotypic variation has been recorded. In addition, significant temporal variation in clinical symptoms and thyroid hormone action has been recorded in affected individuals. Such a phenotypic spectrum has been attributed to as yet unknown environmental/genetic factors (see Beck-Pecoz & Chatterjee, 1994). Apart from serum thyroid hormone levels, thyroid-hormone-responsive variables are often the same in affected and unaffected individuals and most attempts to demonstrate tissue hyposensitivity to thyroid hormones have given contradictory and nonreproducible results (see Beck-Pecoz *et al.*, 1997). Most explanations of the euthyroid-hyperthyroid phenotype associated with RTH have suggested that the high free T4 and T3 levels have overcome the tissue resistance associated with non-functional mutant TRβ receptors and also generated the effects manifested by the TRα receptors. This does not sit easily with the dominant negative effects of such mutant TRβs. However, it is compatible with many thyroid hormone effects, commonly assumed to be mediated by nuclear receptors, instead being mediated by non-nuclear-receptor mechanisms. The attentuated response to exogenous thyroid hormones sometimes recorded in RTH subjects is compatible with the long-known attenuated response to exogenous thyroid hormones associated with hyperthyroidism.

Resistance to the action of thyroid hormones also occurs in situations other than the clinical RTH syndrome. Galo, Unates & Farias (1981) reported the novel finding that the influence of thyroid hormones on rat erythrocyte Ca^{2+}-ATPase activity differed when the rats were fed diets that differed only in their fat composition. When the rats were fed a saturated fat diet, their basal (unstimulated) erythrocyte Ca^{2+}-ATPase activity was less than if the rats had been fed a polyunsaturated diet. The surprising finding was that both T4 and T3 stimulated *in vitro* Ca^{2+}-ATPase activity in the saturated-fat-fed rats but that both thyroid hormones inhibited enzyme activity in the polyunsaturated-fat-fed rats. At *in vitro* concentrations of 10 pM for T3 and 1 pM for T4, the Ca^{2+}-ATPase activity was similar in erythrocytes irrespective of their type of dietary fat. These results showed that not only did thyroid status influence membrane acyl composition (as discussed above) but that membrane acyl composition also has effects on thyroid hormone action.

Other studies also suggest that membrane acyl composition might influence thyroid hormone action. Erschoff (1949) reported that soyabean meal counteracted the thyrotoxic effects of feeding desiccated thyroid tissue to growing rats, that the antithyrotoxic effects were related to the fat component of the soyabean meal, and that the effect was not restricted to soyabean meal but was also present when other fats were used in the diet. His review of the earlier literature (primarily German) indicated that this effect had been known since the 1920s and seemed to be associated with the relative unsaturation of the fats. Whether these early reported influences of dietary fat on the effects of thyroid hormones are mediated by changes in membrane acyl composition is not known. However, later studies have shown that alterations in the acyl composition of the nuclear envelope, by dietary-fat manipulation, also result in changes in T3 binding to

the nuclear envelope (Venkatraman, Lefebvre & Clandinin, 1986).

Fatty acids have been demonstrated to inhibit the binding of thyroid hormones to plasma proteins and to nuclear receptors but such effects are not likely normally to be of *in vivo* importance (Mendel, Frost & Cavalieri, 1986; Mazzachi *et al*., 1992). In mice, a diet enriched with omega-6 polyunsaturates inhibited the stimulatory effects of T4 and T3 on malic enzyme activity but showed no influence on some other hormone effects (Deshpande & Hulbert, 1995).

The interaction between membrane acyl composition and the effects of thyroid hormones is worthy of more detailed examination. For example, ageing is associated with resistance to thyroid hormone action (Mooradian & Wong, 1994) and it is possible that such changes may be mediated by changes in membrane acyl composition with age.

(5) **Future insights from the human genome**

Because nuclear receptors require the presence of TREs at appropriate places in the genome to exert their actions, the complete sequencing of the human genome will allow determination of how many TREs are located in the promoter region of genes and in which genes. It will answer many questions. TREs have been known now for over ten years yet surprisingly few have been described (see Williams & Brent, 1995). A preliminary search of the genome databases in June 1998 for the three types of TRE (Fig. 9) revealed some interesting results. As expected, the vast majority of matches were for the human genome but there were also TREs found in many other types of organisms (including insects, plants and microorganisms). There were curious repeats of palindrome TRE in the genome of *Caenorhabditis elegans*. Some of the matches appeared to be multiple reportings and thus this search may not represent a representative sample of the genome data. The majority (82 %) of TREs found in the human genome were of the direct repeat DR-4 type, with the inverted palindrome (IP-6) type being 13 % and the palindrome type 5 % of total matches. Most matches were not identified as being part of a particular gene but rather on a particular chromosome. TREs were reported for 17 of the 23 human chromosomes. Some of the findings were expected (e.g. deiodinase promoter), whilst other genes such as a chloride channel and a phospholipase were more suprising.

The finding of a TRE associated with a gene need not necessarily mean that its expression is controlled by thyroid hormones unless the TRE is found in an appropriate part of the gene. Some TREs are reported to be in exons and some in introns, whilst others are in promoter regions. Similarly, the finding of a TRE will not by itself mean it is a mediator of thyroid hormone effects. TRs are also required for such thyroid hormone effects to become manifest and therefore the cellular concentration of TRs will give additional insight into their relative importance in different tissues. The fact that TR concentration is greatest in anterior pituitary cells supports the proposal that they are especially important in effects related to the thyroid axis.

An intriguing finding was that the direct repeat TRE (DR+4) was sometimes found associated with another (DR+4) TRE on the other strand some distance away. This may be a type of long-distance palindrome. The complete sequencing of the human genome will allow many questions to be answered.

VI. CONCLUSIONS

(1) The thyroid hormones are very old molecules and appear to be omnipresent among vertebrates. The paradigm that T3 is the 'active' thyroid hormone and T4 is only a prohormone is inadequate. Several iodothyronines are active hormones (namely T4, T3, rT3 and 3,5-T2) and there are several significant pathways of hormone action.

(2) It is proposed that the physical chemistry of these molecules is an important consideration and that several thyroid hormone effects are the consequence of such physical properties. This is additional to, but different from the current paradigm of effects being mediated only by a nuclear receptor mode of action. It agrees with the proposition, recently raised by a review of thyroid hormones in invertebrates, that these hormones may, in many ways, be better thought of in a 'vitamin-like' role than as a classical hormone.

(3) In aqueous solution at physiological pH, approximately 80 % of T4 molecules, but only 10 % of T3 molecules have an ionized phenolic –OH group. Thus one-fifth of T4 and nine-tenths of T3 molecules will be strongly amphipathic. This difference in the relative ionization of the phenolic –OH group is largely responsible for the greater hydrophobicity of T3 compared to T4 and also explains the difference in their relative binding affinities to plasma proteins (generally T4 > T3) and nuclear receptors (T3 > T4).

(4) Iodothyronines are very hydrophobic molecules and their amphipathic nature results in them associating with membranes with the iodine atoms located within the acyl chain region of biological membranes.

(5) The various iodothyronines are formed by sequential monodeiodination of either or both the outer and/or inner ring of T4. Such deiodinations are membrane-associated process and there are three types of deiodinase enzymes (D1, D2 and D3) found in vertebrates. The T3 found in the plasma of vertebrates is produced by deiodination of T4 both in the thyroid follicles and in non-thyroid tissues.

(6) In adult vertebrates, the free plasma concentrations of both T4 and T3 are maintained in the picomolar range whilst the total concentrations of T4 and T3 are in the nanomolar range. The function of the plasma proteins appears to be to ensure a reasonably even and constant distribution of the thyroid hormones throughout the body.

(7) The plasma concentrations of the other iodothyronines are considerably lower than those of T4 and T3 in those vertebrates in which they have been measured. One of the main influences on thyroid hormone concentrations and the various deiodinative pathways appears to be the energy status and food intake of the individual vertebrate being measured.

(8) Although there is considerable variation in the resting metabolic rate of vertebrates there is no correlation between metabolic rate and either plasma T4 or T3 levels. However, the turnover of T4 and T3 in mammals varies with body size in the same way that resting metabolic rate varies. This implies that differences in the secretion rate of thyroid hormones are a response to differences in the metabolic activity of vertebrates rather than the cause of such variations in metabolic rate.

(9) Although thyroid hormones rapidly associate with membranes, because of their amphipathic nature they do not easily cross membrane bilayers. They enter cells by various uptake mechanisms. They diffuse in aqueous solution at rates typical of amphipathic molecules and also diffuse laterally in membranes at rates similar to those of other membrane lipids and at a similar rate to their aqueous diffusion rate. They are found at a variety of locations within the cell and are often located with membranes. It is proposed that they are normal constituents of all membranes in vertebrates. The T4 content of various tissues in the rat is proportional to the phospholipid content of the tissue.

(10) Once associated with membranes they rigidify the fluid membrane bilayer. The mechanism whereby this happens is unknown but it is immediate. The effects of T4 and T3 may be different in this respect and the physical state of the membrane is also a factor.

(11) Thyroid hormones result in an increased degree of unsaturation of membrane acyl chains (especially in the omega-6 PUFAs). The mechanism of this effect is unknown. It is proposed that the normal phospholipid remodelling mechanisms (largely deacylation/reacylation) present in animal cells respond in a 'homeoviscous' manner to the thyroid-hormone-induced membrane rigidification by altering the acyl composition of the membrane. Nuclear receptors may be involved in stimulating some parts of the process. Similarly, their effects on membrane acyl composition may also be connected to the antioxidant activities of thyroid hormones. This is yet to be determined.

(12) T3 binds strongly to nuclear proteins which belong to a superfamily of nuclear receptors. These (TRs) consist of two main types: a TRα and two TRβ receptors (TRβ-1 and TRβ-2) which have different tissue distributions and developmental profiles. TRs act by binding to thyroid response elements (TREs) in the genome and, in turn, activating or repressing transcription of associated genes. There are several types of TRE, which generally have a consensus half-site nucleotide sequence of -AGGTCA-. TREs exist either as a single half-site, a direct repeat of two half-sites separated by four bases, a palindrome separated by six bases or a palindrome not separated by any nucleotide bases. TREs are an evidential requirement for genes that are modulated by thyroid nuclear receptors.

(13) The thyroid hormone axis consists of TRH release from hypothalamic cells, TSH secretion from anterior pituitary thyrotrophs, plasma binding proteins, cellular uptake mechanisms and intracellular deiodinases as well as production of nuclear receptors. This axis has been examined in most detail in adult vertebrates and appears organized as a series of hierarchical regulatory systems that function to maintain a relatively constant level of circulating T4 and relatively constant intracellular T3 levels. The hierarchical nature includes the finding that intracellular homeostasis is maintained in some types of cells at the expense of other cells. The brain appears to be particularly protected in this regard. Almost all the effects of thyroid hormones on the thyroid hormone axis are mediated by nuclear receptors with T3 being the most active intracellular thyroid hormone in this regard; TREs have been

described for many of the important genes involved. One exception is the effect of thyroid hormones on the D2 deiodinase enzyme system where the thyroid hormone effect appears to be on the interaction between the membrane and the intracellular cytoskeleton, with T4 and rT3 being considerably more potent than T3.

(14) Although the effects of thyroid hormones on metabolic rate have been known for some time, the mode of thyroid hormone action until recently remained obscure. Thyroid hormones affect the metabolic activity of cells and tissues by stimulating both the mitochondrial proton leak and ATP turnover of cells. Many cellular processes are increased, including ion channel activity, substrate uptake mechanisms, and the activity of mitochondrial-membrane-bound enzymes. The amount of membranes within cells is also often increased. The vast majority of the effects on metabolic rate can be explained by thyroid-hormone-induced changes in both the amount of membranes and changes in membrane bilayer acyl composition with a consequent increase in the molecular activities of membrane proteins. Ca^{2+} fluxes are increased by thyroid hormones and these effects are relatively immediate and appear to be membrane effects separate from changes in acyl composition. Relatively few effects on metabolism appear to be mediated *via* nuclear receptors. Those that are, such as increased lipogenesis appear to be quantitatively unimportant as sources of increased metabolism, but may be important not only in maintaining fat stores but also in remodelling and manufacture of more membranes.

(15) Thyroid hormones speed up several functions associated with the membranes of nerve and muscle cells. They also stimulate the manufacture of specific isoforms of the sarcoplasmic reticulum Ca^{2+} pump and myosin heavy chains in muscle. Some of these effects are mediated by nuclear receptors. Many other effects, however, appear to be non-nuclear-receptor mediated in that they are too rapid for such a mode of action. The precise mode of action of many of these effects is not known but may be mediated either by direct effects on the membrane bilayer, associated with acyl changes in the membrane bilayer, or are effects due to interaction with specific membrane receptors.

(16) Growth is stimulated by thyroid hormones and part of this stimulation is due to increased secretion of growth hormone (GH) which is a nuclear-receptor-mediated effect. However, the fact that 3,5-T2 also stimulates GH secretion but that TRs have negligible binding affinity for this iodothyronine suggests that other pathways may also be involved. Some of the non-GH-mediated effects may be associated with changes in membrane acyl composition, in that some effects appear to be associated with prostaglandin production.

(17) Thyroid hormones are present from the egg stage through to adulthood and almost all vertebrates appear to have a surge in the plasma T4 levels during development. This is often, but not in all species, associated with a metamorphic event. During development of vertebrates, there are also changes in the deiodinases and TR isoforms, the profiles of which may vary between individual tissues.

(18) The effect of thyroid status on development is often to retard (in the case of hypothyroidism) or accelerate (in the case of hyperthyroidism) particular developmental processes rather than to initiate or stop these changes altogether. This influence on the rate of development of various parts of the vertebrate can be particularly serious during the development of the nervous system. The mode of action of most of these processes is unknown but it is postulated that the recent finding that T4 can influence the interaction between cells and the extracellular matrix is likely to be very important during the development of tissues, especially the nervous system. This finding may also explain the high rT3 and low T3 concentrations that have been measured during the early development of vertebrates (including humans).

(19) Other effects of thyroid hormones include effects on reproduction, defence against viruses and defence against free radicals. The defence against viruses appears to involve two mechanisms. Whilst the mechanisms of the effects on reproductive timing and on learning are unknown, it is possible that they too, involve thyroid hormone effects on the interactions between cells and the extracellular matrix. The role of thyroid hormones as membrane antioxidants has received relatively little attention in recent years but it too deserves more research in light of the findings that at very low concentrations T4 and T3 inhibit the respiratory burst of activated neutrophils. Whether the membrane antioxidant role of thyroid hormones is also associated with its direct effects on membrane fluidity or the thyroid-hormone-induced changes in acyl composition is currently unknown.

(20) A comparison of the relative potencies of thyroid hormone analogues in various effects supports multiple modes of thyroid hormone actions.

(21) The re-examination of results from the early

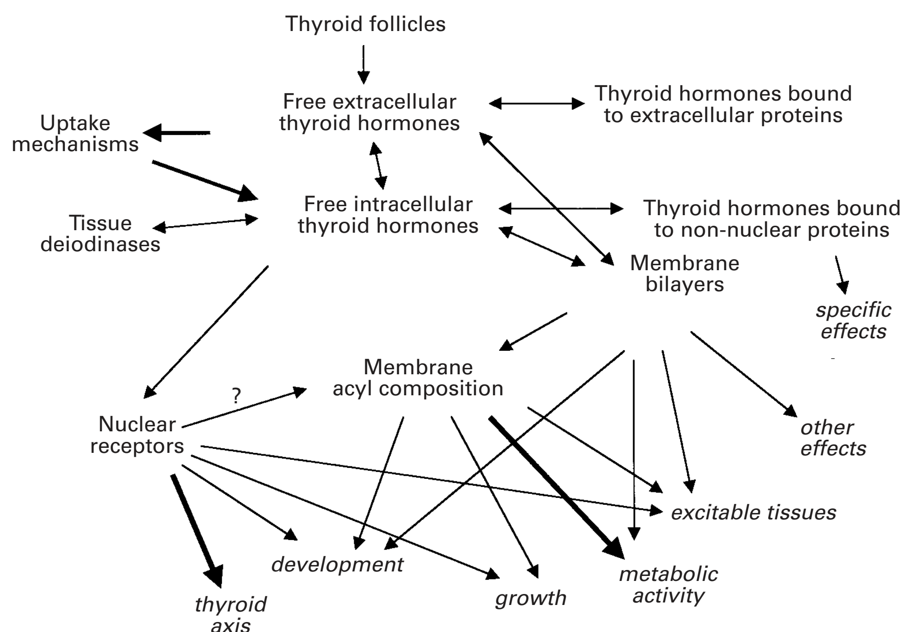

Fig. 14. Schema illustrating the proposed pathways whereby thyroid hormones exert their effects in vertebrates. The effects are shown in italic type. The thickness of the arrows represents the relative importance of the pathway for the particular effects.

1960s that were very influential in the development of ideas concerning thyroid hormone action also support a membrane site of action for thyroid hormones.

(22) 'Knockout' mice, in which the TR isoforms have been inactivated, have been examined. TRβ knockout mice appear normal except for two factors, they exhibit a permanent auditory deficit and are deficient in the thyroid hormone axis in that they are unable to completely suppress TSH release and are thus hyperthyroid. Two strains of TRα knockout mice were produced. Those that are only deficient in TRα-1 also appear normal except that they have reduced TSH levels and are mildly hypothyroid. Another strain of knockout mice in which both the TRα-1 and the non-thyroid-hormone binding, but related, receptor c-erbAα-2 are non-functional shows the most extreme abnormalities. These mice appear normal until the time of the developmental T4 surge. At this time they develop extreme hypothyroidism, fail to grow or develop normally and generally die. When affected individuals were given a short temporary burst of exogenous thyroid hormone several became euthyroid, resuming normal development and surviving for a considerable time without exogenous thyroid hormone but instead now making their own. The relative lack of effects in these knockout mice strains suggests that there may be a certain amount of redundancy between TRα and TRβ with one receptor isoform capable of substituting if the other is absent. However, there were no compensatory increases in expression of the other receptor in either TRα or TRβ knockout mice. The results show the importance of both of these receptors in the homeostatic system that maintains normal thyroid hormone levels in the adult. They also suggest that the non-thyroid-hormone-binding c-erbAα-2 receptor may also have an important role in initiating the developmental surge of T4 concentration.

(23) The clinical syndrome of 'resistance to thyroid hormone' is due to mutations in the TRβ receptor in those humans affected. The considerable phenotypic variation found for this syndrome is compatible with multiple pathways of thyroid hormone action. Results from animal studies suggest that factors such as dietary fat composition (which can influence membrane acyl composition) can also modulate thyroid hormone effects.

(24) In conclusion, this review suggests that the effects of thyroid hormones can be mediated by a number of pathways and proposes that there are several modes of action: (i) the binding of T3 to thyroid nuclear receptors is an important pathway of

hormone action, especially in the control system that maintains relatively constant hormone levels in adult vertebrates, it is also important in some other effects, (ii) due to their physical properties, thyroid hormones are normal constituents of membranes and correspondingly influence the properties of these membranes and their function; they also cause changes in the acyl composition of membranes which, in turn, influence the behaviour of proteins associated with such membranes and this is an important mode of stimulation of metabolic activity, (iii) there are additional membrane effects about which little is known, such as interaction with the extracellular matrix and probable binding to proteins which can be regarded as receptors for non-nuclear-mediated effects. The proposed pathways whereby thyroid hormones exert their effects are summarised in Fig. 14.

(25) Although study of the thyroid gland and the effects of its hormones has a relatively long history, much still remains to be investigated before we know the full picture of the connections between these hormones and their observed effects.

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