

The Role of Thyroid Hormones in the Growth of Striated Muscles in the Neonatal Rat

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Abstract

The effects of the normal increases in endogenous thyroid hormones on the growth of the diaphragm and the ventricles and atria of the heart have been studied 20 days *post partum*. The thyroid status of these neonatal rats was also experimentally modified to either prevent their normal increase (i.e. hypothyroid) or to induce both a precocious and greater rise in their circulating levels (i.e. hyperthyroid). The impact of these perturbations on the rates of protein turnover and growth of the same 3 striated muscles was studied. Insufficient thyroid hormones significantly retarded the growth of the whole animal and the 3 muscle types; the diaphragm and combined ventricles being more severely affected than the atria. In all muscles the rates of protein synthesis were significantly inhibited, with these correlating more closely with decreases in the rates of synthesis per ribosome rather than changes in ribosomal capacity. In contrast, hyperthyroidism resulted in accelerated muscle growth, with the increases in protein, RNA and DNA being more pronounced in the atria and the ventricles than in the diaphragm. Protein synthetic rates were increased in all muscles, largely as an increase in ribosomal activity. Clearly thyroid hormones are important in the growth of neonatal muscle.

Key Words: hypothyroid, hyperthyroid, protein synthesis, muscle growth, atrophy, diaphragm, atria, ventricles.

BAM 3 (2): 85-92, 1993

Adult rat and human striated muscles appear to be highly responsive to the level of circulating thyroid hormones. Inappropriate maintenance of the euthyroid state results in qualitative and quantitative changes in the musculature. Raised thyroid concentrations in both man and animals leads to cardiac hypertrophy and an enhanced cardiac output as a result of the increased frequency and force of contraction [7]. Under such conditions the rate of cardiac protein synthesis is enhanced, together with a smaller antagonistic increase in protein degradation. The net result, however, is protein accretion and hypertrophy [30]. The fast myosin heavy chain (i.e. α MHC) of the ventricles is up regulated, clearly demonstrating the ability of these hormones to modulate expression in this multigene family [14].

In skeletal muscle hyperthyroidism usually induces muscle atrophy and generalised weakness. Although protein turnover is accelerated as in the hyperthyroidic heart, in skeletal muscle the increased rate of protein breakdown exceeds that of protein synthesis, resulting in a net loss of

muscle protein [2, 19]. In all skeletal muscles, regardless of type, the proportion of type II fibres increases [17, 28], as does the accumulation of the mRNA for the IIa and IIb MHC [15, 18, 25].

In hypothyroidic animals cardiac hypodynamia and atrophy occur principally as a result of decreased rates of protein synthesis with no change in protein degradation [16]. In the ventricles of the hypothyroid rat the β -MHC is up regulated at the expense of the α -MHC. In the short term this switch in gene expression and the ensuing slowing in the velocity of shortening is energetically more favourable. It does not, however, occur in either the rat atria or in the human ventricles where, as part of a species difference, the β -MHC is already preferentially expressed in the euthyroid state [14, 18, 25].

In the skeletal musculature a deficiency of T_3 and T_4 causes the rates of both protein synthesis and degradation to decrease, but in such a manner as to create a net catabolic state [9]. In contrast to hyperthyroidism insufficient thyroid hormones increases the concentration of the mRNA

for the slow MHC and eventually the proportion of type I fibres at the expense of type II fibres [18].

In contrast to the adult animal, much less is known about the impact of thyroid hormones on the growth of striated muscles in the neonate. During the suckling period the rat experiences a rise in the endogenous free T_3 and T_4 concentrations [32], with these increasing from birth to peak at about 22 days *post partum*. Thereafter, they decline again. Striated muscles are known to contain functional thyroid hormone receptors at this age and like several other body tissues grow rapidly during this time [31]. We have, therefore, designed experiments to ensure that neonatal rats experienced either a precocious increase or no change in plasma T_3 and T_4 concentrations to coincide with the normal endogenous release of these hormones. The impact of these perturbations in thyroid hormone status on the growth of the atria and ventricles of the heart and on the diaphragm were investigated. These experiments clearly indicate that the rates of protein turnover and growth of these striated muscles are acutely sensitive to thyroid hormones during early postnatal life.

Materials and Methods

Female Wistar rats weighing 310 ± 15 g were mated with proven males; pregnancy being determined by palpation of the uterus from day 11 onwards. The pregnant dams were then subdivided into 4 groups; 2 groups destined to produce control (i.e. euthyroid) neonates, the third hypothyroid and the fourth group hyperthyroid neonates. All animals had free access to water and were fed *ad libitum*. At birth all litter sizes were reduced to 12 pups. Changes in thyroid status were achieved as follows.

Neonatal hypothyroidism was induced by adding propylthiouracil (PTU) to the drinking water (200 mg/l) of both pregnant (from day 12 of gestation) and lactating dams, through to weaning. Appropriate euthyroid controls (i.e. non-injected neonates, group I) were used for comparison.

Precocious hyperthyroidism was induced by administering (S.C., maximum vol. of 200 μ l) T_4 from day 3 through to day 20 *post partum*, at a dose of 0.1 μ g/g of body wt. Appropriate controls (i.e. euthyroid, group II) received a similar volume of the 0.9% saline vehicle to allow for any stress caused by the daily injections.

All experimental procedures were standardised and performed between 09.00 and 12.00 h to minimise possible diurnal variations. Protein synthesis was measured *in vivo* after an injection of a large dose of phenylalanine to flood the precursor pools [10]. This injection was administered (I.P.) to each neonatal rat and contained 150 μ mol of the amino acid, including 65 μ Ci of L-(4- 3 H) phenylalanine (sp. radioactivity 24 Ci/mmol; from The Radiochemical Centre, Amersham, Bucks., UK), in 1 ml of 0.9% NaCl per 100 g body wt. All animals were decapitated 10 min after commencing the injection. Thoracic and abdominal cavities were immediately opened and the whole animal submerged in ice-cold NaCl. The heart was isolated and

the atria and ventricles separated. These, together with the diaphragm, were dissected out within a few seconds and frozen in liquid N_2 .

Frozen individual tissues were pulverized between chilled metal plates. Tissue samples were then immediately homogenized in ice-cold 0.3M-HClO₄ in a ground-glass homogenizer. The specific radioactivities of the phenylalanine both in the 'flooded' tissue pool(s) and covalently bound in protein were measured in the homogenates of all muscles by the method described by Garlick *et al.* [10]. This involved the prior hydrolysis of the washed protein pellets in 6M-HCl at 110°C for 24 h and the conversion of phenylalanine into β -phenethylamine [10]. All measurements of radioactivity were made in a LKB scintillation counter (efficiency for 3 H of 25%) in Opti-phase (Pharmacia) scintillant with the use of an external standard. The fractional rate of synthesis (i.e. k_s , the percentage of the protein mass synthesized per day) was calculated from

$$k_s = \frac{S_B}{S_A t} \times 100$$

where S_A and S_B are the specific radioactivities of phenylalanine in the free tissue pool (i.e. intracellular and extracellular) and protein respectively, and t is the time in days.

Protein was measured in the same tissue preparations by the method of Lowry *et al.* [24] by using bovine serum albumin (Sigma, Kingston upon Thames, Surrey, UK) as standard. RNA and DNA were extracted and measured as previously described [1, 12].

Radioimmuno assays for T_3 & T_4

Samples (50 μ l) of mixed whole blood were collected at the time of killing and total T_3 and T_4 concentrations measured using an inhouse modification of the method described by Gharib *et al.* [11].

Statistical analysis

The various parameters associated with muscle growth are presented as means $\pm$ SEM. Student's unpaired two-tailed t-test was used to establish the level of significance between the values of the control and thyroid manipulated groups of animals.

Results

Our experimental aims were to i) prevent the normal postnatal surge of thyroid hormones, thereby creating a state of hypothyroidism, ii) advance and elevate this endogenous surge to induce a state of hyperthyroidism, and iii) determine the effects of these changes in thyroid status on the growth of 3 striated muscles during early postnatal life.

Between the end of gestation and 20 days *post partum* the concentrations of T_3 and T_4 normally rise from 0.4 ± 0.03 to 2.4 ± 0.2 mmol/l and from 21 ± 2 to 85 ± 3 mmol/l, respectively. After treatment with PTU this early postnatal

surge of T_4 was completely abolished and the of T_3 reduced to approximately 40% of normal. Hence, by 20 days *post partum* the circulating T_3 and T_4 levels had decreased by 58 and 72% respectively (Table 1), clearly indicating that hypothyroidism had been achieved.

In contrast, daily injections of T_4 from birth markedly increased the circulating values of both T_3 and T_4 (Table 1), inducing a state of hyperthyroidism. The subsequent effects of these changes in thyroid status were studied in relation to the growth of the diaphragm, and the atria and ventricles of the heart (Tables 2-6).

Two different groups of control animals (i.e. groups I & II) were examined to compensate for the different routes of administering the PTU and exogenous T_4 . Although seldom significantly different, the wet weights, protein and nucleic acid contents of all 3 muscle types were consistently lower in the injected control pups (i.e. group II, Tables 2 & 3). These observations, together with slightly higher levels of corticosterone measured in the plasma of the injected animals (i.e. 225 ± 12 compared with 178 ± 48 ng/ml), suggest some injection-related, and growth retarding, stress which necessitated a separate group of controls.

By the 20th day *post partum* significant differences were apparent in the thyroid manipulated neonates. The hypothyroid state was accompanied by significant growth retardation of the pups (Table 2). This decrease in body weight (34%) was paralleled by even greater losses in the weight of both the diaphragm and ventricles (Table 2), but less growth retardation of the atria (24%), liver (28%) and lungs (12%). These changes in tissue weight could not be explained by a simple repartitioning of water, since the protein masses and nucleic acid contents consistently followed these trends within the 3 muscle types (Tables 2 & 3). Furthermore, the proportion of protein in the wet weight was not significantly altered at 12-15%.

Table 1. Changes in total plasma thyroid hormones in the 20 day neonates after experimental manipulation.

	T_3 (n mol/l)	T_4 (n mol/l)
Control	2.4 ± 0.15 (n = 8)	85 ± 3 (n = 8)
Hypothyroid	$1.0^{**} \pm 0.04$ (n = 11)	$24^{**} \pm 1$ (n = 10)
Hyperthyroid	$6.4^{**} \pm 0.26$ (n = 4)	$351^{**} \pm 19$ (n = 6)

The concentration of total tri-iodothyronine (T_3) and thyroxine (T_4) were measured in the plasma of 20 day old neonates by radioimmunoassays after the induction of hypo- and hyper-thyroidism. Values are presented as means $\pm$ SEM for the number of samples shown above.

**P < 0.01

In contrast, hyperthyroidism produced essentially the opposite changes, but with some interesting exceptions. After 17 days of excess T_4 these pups were slightly (15%), but not significantly, larger than the injected controls. All 3 muscles were significantly heavier than their equivalent euthyroid controls, with this consistently reflected through greater increases in their protein, RNA and DNA contents (Tables 2 & 3). Interestingly the combined atria were now the most, and the diaphragm the least, responsive of the muscles to the elevated circulating T_3 and T_4 hormones.

To explain the alterations in muscle growth (i.e. protein accumulation) in response to the changes in thyroid status, average rates of protein synthesis were measured *in vivo* in each of the 3 types of striated muscles (Tables 4-6).

The fractional rate of synthesis (i.e. %/day) in the normal atria was approximately 65% higher than that of the ventricles; the rate in the diaphragm lying between these two values (Tables 4-6). Despite these differences, the ribosomal capacities (ie RNA/protein) of all three muscles were very similar, leaving the higher synthesis rate per ribosome (73%) in the normal atria (Table 5) as the principal difference to that found in the ventricles (Table 6).

Without exception, hypothyroidism decreased that rate of protein synthesis in all 3 muscles; the larger changes (e.g. in the diaphragm; Table 4) generally corresponding with the greater degree of growth retardation. These reduced rates of synthesis in the diaphragm and ventricles correlated with lower synthesis rates per ribosome, rather than changes in ribosomal capacity (Tables 4 & 6). In the atria both of these aspects of the translation process appeared to be reduced to similar extents (Table 5). Although hyperthyroidism significantly increased the total rates of protein synthesis in all three muscles (Tables 4-6), the fractional rate was only significantly elevated in the ventricles, with this correlating with an increase in the rate of synthesis per ribosome (Table 6). The magnitude of these changes in protein synthesis, relative to the extent of the muscle hypertrophy (Table 2), strongly suggests that the rate of protein degradation was decreased in all 3 muscles in the presences of the excess thyroid hormones.

Discussion

The possible involvement of thyroid hormones in the rapid growth of neonatal striated muscles was examined by the successful induction of hypo- and hyper- thyroidism.

PTU administration to the dams during suckling effectively prevented the normal neonatal rise in plasma T_3 and T_4 (Table 1). It has previously been shown that PTU is capable of crossing placental and mammary gland membranes [23]. Hence, our treatment was initiated on day 12 of gestation to pre-empt any synthesis and storage of thyroid hormones by the perinates; events which are normally initiated in the fetal rat around 17 to 18 days of gestation [8]. As rat milk is known to contain T_3 and T_4 [22] the treatment of the dams was continued during suckling to minimise the possible passage of thyroid hor-

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Table 2. Changes in the weight and protein mass of the 20 day neonate and three of its striated muscles in response to altered thyroid status.

	Whole Body wt (g)	Diaphragm wt (mg)	protein (mg)	Atria wt (mg)	protein (mg)	Ventricles wt (mg)	protein (mg)
Control (I)	44 ± 1.7	169 ± 8.7	20 ± 1.5	23 ± 0.7	3.1 ± 0.1	261 ± 15	34 ± 2.2
Hypothyroid	2.9 ± 1.2	88 ± 5.5	12 ± 1.2	18 ± 1.6	2.8 ± 0.1	132 ± 8.8	20 ± 1.6
% decrease	34***	48*	42**	24***	10*	50***	42***
Control (II)	40 ± 3.3	142 ± 8.4	18 ± 1.3	24 ± 2.4	2.6 ± 0.1	209 ± 7.9	31 ± 1.9
Hyperthyroid	46 ± 1.2	164 ± 5.5	23 ± 1.0	44 ± 1.7	6.3 ± 0.7	343 ± 13	58 ± 0.3
% increase	15	16*	29**	86***	142***	65***	88***

The wet weights and protein masses [24] of the whole neonate and 3 of its striated muscles were measured (means ± SEM) in 8 saline injected (II) and non-injected (I) control animals and the same number of hypo and hyperthyroid neonates 20 days *post partum*. The whole diaphragm was isolated from each animal and the heart separated into the combined left and right atria and ventricles.

(*P < 0.05; **P < 0.01; ***P < 0.001)

Table 3. Nucleic acid contents of 3 striated muscles in eu-, hypo-, and hyper-thyroid neonates.

	Diaphragm RNA (µg)	DNA (µg)	Atria RNA (µg)	DNA (µg)	Ventricles RNA (µg)	DNA (µg)
Control (I)	371 ± 31	307 ± 19	66 ± 2.0	51 ± 0.3	709 ± 16	637 ± 25
Hypothyroid	198 ± 8	160 ± 11	48 ± 2.0	38 ± 0.1	435 ± 25	451 ± 30
% decrease	47**	48**	27***	26***	39***	29**
Control (II)	315 ± 20	284 ± 12	58 ± 2.0	46 ± 1.1	641 ± 55	593 ± 19
Hyperthyroid	392 ± 15	303 ± 11	123 ± 6.3	73 ± 2.9	1137 ± 55	822 ± 34
% increase	24*	7	111**	59***	77***	39**

The nucleic acids of the 3 muscles described in Table 2 were extracted and measured [1, 12] and the results presented as means ± S.E.M

(*P < 0.05; **P < 0.01; ***P < 0.001)

mones from the mother to the offspring. PTU acts by blocking both the synthesis of T₄ by the thyroid gland and its peripheral conversion into T₃ (Cooper *et al*, 1984). It poses no known risks to the fetus, neonate or adult and as such has been used in humans to treat pregnant women suffering from Grave's disease [4].

The PTU-induced hypothyroidism caused growth retardation of the neonate, the 3 striated muscles studied (Tables 2 & 3) and the lungs, liver and kidney (J. Holt, J.P. Canavan & D.F. Goldspink, 1993-unpublished informa-

tion). In all of these tissues the fractional and total rates of protein synthesis were significantly decreased (Tables 4-6). The largest changes in synthesis occurred in the diaphragm (Table 4), which was the most profoundly growth retarded of the 3 muscles (Table 2 & 3). The next was the ventricles, with the atria being the least affected by the deficiency of T₃ and T₄ (Tables 2 & 3).

Similar decreases in the rates of protein synthesis have been measured in striated muscles of adult hypothyroid animals [2]. However, in the neonatal muscles studied here

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Table 4. Rates of protein synthesis in the diaphragm of eu-, hypo- and hyper-thyroid neonates.

	(%/day)	Protein synthesised (mg/day)	(mg/mg of RNA/day)	Ribosomal Capacity (mgRNA/g protein)
Control (I)	24 ± 1.9	5.0 ± 0.7	13 ± 0.8	18 ± 0.6
Hypothyroid	11 ± 0.5	1.3 ± 0.2	6.3 ± 0.6	17 ± 0.9
% decrease	56***	74**	52***	6
Control (II)	17 ± 1.0	3.0 ± 0.2	9.4 ± 0.4	18 ± 0.4
Hyperthyroid	18 ± 1.1	4.2 ± 0.3	11 ± 0.4	17 ± 0.9
% increase	10	40**	14*	3

The rates of protein synthesis were measured *in vivo* [10] in the diaphragms of the neonatal rats described in Table 2. The total rates of synthesis are the products of the appropriate fractional rates (i.e. %/day) and protein masses (Table 2). Ribosomal capacities (i.e. RNA/protein) and synthesis rates per ribosome (i.e. synthesis/RNA) were derived from the appropriate protein and RNA contents provided in Tables 2 & 3, respectively.
(*P < 0.05; **P < 0.01; ***P < 0.001)

Table 5. Protein synthesis rates in the atria of eu-, hypo- and hyper-thyroid neonates.

	(%/day)	Protein synthesised (mg/day)	(mg/mg of RNA/day)	Ribosomal Capacity (mgRNA/g protein)
Control (I)	21 ± 0.02	0.7 ± 0.01	9.9 ± 0.2	21 ± 0.5
Hypothyroid	16 ± 1.4	0.4 ± 0.1	8.2 ± 1.2	18 ± 0.2
% decrease	25*	39***	17	15**
Control (II)	24.5 ± 3.2	0.7 ± 0.1	13 ± 2.5	22 ± 0.3
Hyperthyroid	23.7 ± 0.5	1.5 ± 0.1	12 ± 0.7	20 ± 1.5
% change	-4	+102***	-5	-8

The same measurements were made on the combined atria, as described for the diaphragm in Table 4.
(*P < 0.05; **P < 0.01; ***P < 0.001).

the suppression of synthesis rates appeared to be linked to decreases in ribosomal activities (Tables 4-6), unlike the adult where it correlated with a reduction in ribosomal capacity [2]. The extent of the growth retardation in the 3 muscles was less than the decreases in the synthesis rates, suggesting that the rates of protein degradation probably decreased also. This would be consistent with the effects of thyroid insufficiency on skeletal muscles of adult animals [20]. Thyroid hormones are thought to activate lysosomal and Ca²⁺-activated (i.e. calpains) proteinases in skeletal muscle, hence removal of these hormones would be expected to decrease proteolysis [20]. In addition to

these direct actions of T₃ and T₄ on striated muscle growth, other indirect effects may be operating.

Low T₃ levels in neonatal animals can lead to reduced transcription of the growth hormone gene, leading to a concomitant decline in the circulating level of insulin-like growth factor, IGF_I [33] which is known to stimulate protein synthesis in fully differentiated skeletal muscle [20]. Hence, the observed decreases in muscle protein synthetic and growth rates in the hypothyroid pups may arise, at least in part, from the depressed secretion of growth hormone and growth factors. T₃ is, however, capable of promoting the growth of striated muscle in hypophysectomized animals where growth hormone is absent

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Table 6. The effects of altered thyroid status on protein synthesis in the combined ventricles.

	(%/day)	Protein synthesised (mg/day)	(mg/mg of RNA/day)	Ribosomal Capacity (mgRNA/g protein)
Control (I)	14 ± 0.4	4.7 ± 0.4	6.6 ± 0.5	21 ± 0.9
Hypothyroid	11 ± 0.6	2.2 ± 0.6	4.9 ± 0.2	23 ± 1.1
% decrease	18**	53**	25**	+6
Control (II)	14 ± 0.2	4.3 ± 0.3	6.6 ± 0.2	21 ± 0.6
Hyperthyroid	16 ± 0.3	9.2 ± 0.3	8.1 ± 0.3	20 ± 0.5
% increase	14***	114***	23**	-6

The same measurements were made on the combined ventricles, as for the diaphragm (Table 4).
(**P < 0.01; ***P < 0.001)

and IGF₁ levels extremely low [16]. Hence, it is probable that low thyroid hormone levels *per se* do contribute significantly to the reduced growth in the hypothyroid animals.

Low T₃ and T₄ concentrations in early life can also impair the development of the CNS. This, together with a decrease in the number of β-adrenergic receptors, could affect the work load and output of the heart and the activity of the diaphragm.

In our studies single daily injections of T₄ from day 3 onwards successfully advanced and elevated plasma T₃ and T₄ levels (Table 1). We chose to administer T₄ rather than the biologically more active T₃ because of its longer half-life. Although the circulating concentrations of the thyroid hormones were very high at 20 days *post partum*, neither this dosage nor the injections themselves appear to have produced any serious thyrotoxic or Corticosterone induced side effects as the body weight gains were normal (Table 2). This contrasts with the postnatal growth retardation which occurs when the hypothalamic-pituitary axis is deranged as a consequence of administering pharmacological doses of T₄ [27].

The temporal advance of the normal neonatal rise in T₃ enhanced the accumulation of protein and nucleic acids in the atria and ventricles of the heart, and to a lesser extent in the diaphragm also (Tables 2 & 3). These differences between the muscles may be explained, at least in part, by T₄-induced increases in cardiac work load. In the rat, when heterotopically transplanted hearts (i.e., unloaded) are subjected to the same 2 weeks of hyperthyroidism as the recipient's own heart (i.e. loaded) only the latter undergoes hypertrophy. In fact, the transplanted heart often undergoes atrophy. Despite these differences in growth both hearts experienced similar increases in the α-MHC isoform [21]. Hence, the switches in gene expression, but not

the hypertrophy appear to reflect the direct actions of these hormones on these striated muscles [18, 21].

The large increases in DNA in the atria and ventricles could be non-myocyte in origin, reflecting the proliferation of interstitial cells. It is, however, generally agreed that thyroid hormones are not directly mitogenic [3]. Furthermore, the T₃-induced cardiac hypertrophy in adult animals is not associated with significant fibroblast proliferation or subsequent deposition of collagen [29]. Thus, in our study the T₄-induced cardiac growth probably represents myocyte hypertrophy. Rather than interstitial cell proliferation the increase in DNA content probably represents an increase in myocyte binucleation, this being a function of cardiac size. No significant increase in DNA was found in the diaphragm in the presence of excess T₄ (Table 3).

Hyperthyroidism in the neonates increased both the fractional and total rates of protein synthesis in all 3 muscles (Tables 4-6). Protein synthesis rates are similarly elevated in skeletal muscle and the ventricles of the heart [2] in adult rats. However age-related differences seem to exist in the site of control of protein synthesis. In the adult the enhanced synthetic rates arise from increases in the ribosomal capacity, while in the neonate they correlate with increased ribosomal activities (Tables 4-6). Thyroid hormones have previously been shown to accelerate the elongation stage of translation in the liver [5].

The overall effect of thyroid hormones in adult skeletal muscle appears to depend on the degree of the hyperthyroidism induced. Protein synthetic rates are elevated whether the hyperthyroidism is defined as physiological or pharmacological [13]. Only in the latter situation however are the rates of protein degradation substantially increased (Goldberg, 1980). Thus, during moderate hyperthyroidism muscle growth can occur whilst in severe hyperthyroidism atrophy will occur, with the increase in protein catabolism exceeding that of synthesis. In the neonatal rat the dia-

phragm is predominantly a fast muscle [26]. Its accelerated growth in the presence of excess T_4 was primarily due to an increased rate of protein synthesis, with little or no change in protein degradation (Table 4). In contrast to the additional growth expressed in the striated muscles, the growth of the lungs and liver was unaffected by neonatal hyperthyroidism (J Holt, J P Canavan & DF Goldspink, 1993 - unpublished information). Thus not all tissues of the body are as acutely sensitive to changes in thyroid status as the striated muscles.

Acknowledgements

The authors are grateful to the National Heart Research Fund for their financial support of this work and Dr C Chapman for his assistance with the radioimmune assays.

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