Role of L-Thyroxine in Nuclear Thyroid Hormone Receptor Occupancy and Growth Hormone Production in Cultured GC Cells

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Abstract

The contribution of L-thyroxine (T_4) to nuclear thyroid receptor occupancy was studied in GC cells incubated with concentrations of 3,5,3'-triiodo-L-thyronine (T_3) and T_4 that resulted in free iodothyronine levels similar to those in serum of euthyroid rats. T_4 accounted for 5.4–10% of the occupied receptors: T_3 derived from T_4 [$T_3(T_4)$] and T_3 added to medium accounted for the remainder of receptor occupancy. Incubation with increasing medium free T_4 resulted in a progressive increase in the contribution of T_4 and $T_3(T_4)$ to receptor occupancy. In incubations with 3.6-fold increased medium free T_4, T_4 accounted for 20.4%, and $T_3(T_4)$ for 40.3% of receptor occupancy. These occupancy data and the experimentally determined K_a of thyroid receptor for T_3 and T_4 allowed calculation of nuclear free iodothyronine concentrations. Nuclear free T_3 was 3-6-fold greater than medium free T_3 and medium free T_4 was 12–19-fold greater than medium free T_4. When GC cells were incubated with decreased medium free T_3 and physiological medium free T_4, both nuclear receptor occupancy and growth hormone production decreased as well. However, a twofold increase in medium free T_4, in the presence of decreased medium free T_3, restored receptor occupancy and growth hormone production to or near control values. These findings establish a role for T_4 in addition to $T_3(T_4)$ in nuclear receptor occupancy and biological activity in rat anterior pituitary tissue both in physiologic conditions and when medium free T_4 is raised. The findings may have relevance to the sick euthyroid thyroid syndrome in which free T_4 may be increased in some patients who have decreased serum free T_3. (*J. Clin. Invest.* 1991. 88:1291–1299.) Key words: L-triiodothyronine (T_3) • L-thyroxine (T_4) • nuclear free T_3 and free T_4 • sick euthyroid syndrome • type II 5'-T_4 monodeiodinase

Introduction

L-Thyroxine (T_4) was reported in 1948 to be the circulating thyroid hormone by Taurog and Chaikoff (1). The discovery of 3,5,3'-triiodo-L-thyronine (T_3) in 1952 (2) and demonstration that T_3 had greater biological activity than T_4 (3) led Gross and Pitt-Rivers (2) to suggest that effects of T_4 might result from deiodination to T_3. However, experimental methods were not sufficiently sensitive to test this hypothesis at that time (4, 5). Measurements of plasma T_3 became available in 1970 (6) and investigators rapidly demonstrated that most of the extrathyroidal T_3 pool in humans and rats was generated by deiodination of T_4 (7–9). Moreover, conversion of T_4 to T_3 was required for a major portion of the apparent activity of T_4 (10). These findings and the discovery that the nuclear thyroid hormone receptor had a greater affinity for T_3 than for T_4 (11, 12) suggested that T_4 was mainly a prohormone, effecting biological responses by means of generated T_3.

In euthyroid rats, T_3 that is locally generated from T_4 [$T_3(T_4)$][1] within brain and anterior pituitary constitutes the principal source of nuclear T_3, whereas nuclear T_3 in liver and kidney is derived mainly from exchangeable plasma T_3 [$T_3(T_3)$] (13, 14). The role of T_4 itself in occupancy of nuclear thyroid hormone receptors has not been established in anterior pituitary. Studies in euthyroid rats suggested that T_4 constituted 10–15% of the endogenous iodothyronines specifically associated with thyroid nuclear receptors in liver and kidney (15) but was not detected in anterior pituitary (16, 17). T_4 was shown to have intrinsic biological activity by Samuels et al. (18), who showed that T_4 induced growth hormone (GH) production and thyroid nuclear receptor down-regulation in GH_1 cells in the absence of appreciable conversion of T_4 to T_3. More recently, others have shown that GH_1 cells do generate appreciable amounts of T_3 from T_4 (19).

Because interactions between iodothyronines and nuclear thyroid receptors are considered to initiate thyroid hormone action, the foregoing studies suggest that T_4 itself effects 10–15% of the biological activity of thyroid hormones in liver and kidney. Although T_4 affects biological activity in anterior pituitary by means of $T_3(T_4)$, these reports (16–19) also suggest that the role of T_4 itself in thyroid hormone action in anterior pituitary is not clearly defined. The issue is important for understanding both the action of thyroid hormones under physiologic conditions and during the alterations in plasma thyroid hormone concentrations that occur in patients with nonthyroidal disease. Unlike normal individuals, patients without thyroid disease who have an acute nonthyroidal illness are characterized by a decrease in the serum free T_3 concentration and either a normal or increased concentration of the serum free T_4 (20–22). Data concerning the biological impact of the normal or raised concentrations of free T_4 in this acute clinical setting have not been reported. It is not known, for example, whether

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1. *Abbreviations used in this paper:* GH, growth hormone; MFT_3, medium free T_3; MFT_4, medium free T_4; NFT_3, nuclear free T_3; NFT_4, nuclear free T_4; NS-DME, DME containing 10% normal serum; RT-DME, DME containing 10% resin-treated calf serum; $T_3(T_4)$, T_3 derived from T_4 monodeiodination; $T_3(T_3)$, T_3 derived from medium T_3; TR, nuclear thyroid receptor.

the high concentration of free T_4 in some patients with acute illness initiates biological activity which offsets the decrease in activity anticipated as a consequence of decreased concentrations of serum free T_3.

Because of these uncertainties, we have investigated the role of T_4 in the occupancy of nuclear thyroid hormone receptors using cultured GC cells, a well-characterized rat GH-producing cell line that is responsive to thyroid hormones. We have studied nuclear thyroid hormone receptor (TR) occupancy by T_4 and T_3 during incubation of GC cells in medium which contains free T_4 and free T_3 concentrations similar to those in euthyroid rats. Studies were also done in cells maintained in medium in which free T_4 and free T_3 were altered in a manner that is similar to patients with nonthyroidal disease.

Methods

Materials. L-T_3, 3,5-diiodo-L-thyronine, L-T_4, D-T_3, D-T_4, and Dowex 1×8, 400-mesh anion exchange resin were purchased from Sigma Chemical Co., St. Louis, MO. T_4 was purified by paper chromatography (22). The content of T_3 in the purified T_4 was assessed by supplementing iodothyronine-depleted human serum (23) with the purified T_4 and measuring T_3 using a Clinical Assays Kit (Travenol Laboratories, Inc., Cambridge, MA). Using these methods, the purified T_4 preparations contained < 0.05% T_3. Dulbecco's modified Eagle's medium (DME), heat-inactivated calf and horse serum, and trypsin-EDTA were purchased from Gibco Laboratories, Grand Island, NY. Iopanoic acid was purchased from Chemical Dynamics Co., South Plainfield, NJ. Reagents for measurement of rat GH by radioimmunoassay (RIA) were a gift of the National Hormone and Pituitary Program, National Institute of Digestive Diseases and Kidney, National Institutes of Health, Bethesda, MD. Carrier-free Na[^{125}I]iodide and Na[^{131}I]iodide were obtained from New England Nuclear, Boston, MA. ^{131}I-labeled T_3 was synthesized from carrier-free [^{131}I]iodide and 3,5-diodothyronine, and ^{125}I-labeled T_4 was synthesized from carrier-free [^{125}I]iodide and T_3 according to the methods of Kochupillai and Yalow (24). Iodothyronines were purified as previously described (22). [^{131}I]T_3 had a specific activity of 2.13 Ci/μmol (3,280 μCi/μg) and was > 95% pure as assessed by paper chromatography (22) [^{125}I]T_4 had a specific activity of 2.13 Ci/μmol (2,750 μCi/μg) and contained < 0.1% of [^{125}I]T_3 as assessed by paper chromatography (22). In that Freake et al. (25) reported that racemization of L-T_3 may occur during iodination, we measured the racemic contamination of L-[^{125}I]T_3 and L-[^{125}I]T_4 with D-isoforms by high-performance liquid chromatography (HPLC) using the method of Hay et al. (26) (Fig. 1). < 6% [^{125}I]D-T_3 and < 3% D-[^{125}I]T_4 were found in the respective ^{125}I-labeled preparations of the L-isomers. Because of these results, we considered that the radiolabeled L-isomers were all in the L-form and made no corrections for the putative small degree of racemization that was observed.

Free T_4 and free T_3 concentrations in rat serum. Male CD (Sprague-Dawley) rats were obtained from Charles River Laboratories, Inc., Wilmington, MA. The rats were housed in a vivarium, fed Wayne Laboratory rat diet (Allied Mills, Inc., Memphis, TN), and allowed tap water ad lib. Rats generally weighed between 200 and 260 g at the time they were studied. After the animals had been maintained in the vivarium for at least 1 wk, groups of rats were killed by exsanguination through the abdominal aorta under light ether anesthesia. Serum was obtained by centrifugation. The animal study protocol was approved by the Institutional Animal Care and Use Committee (Protocol 85-10-213).

Rat serum T_4 was measured with a Clinical Assays Kit (Travenol Laboratories, Inc.). Serum T_3 was determined with a T_3 RIABEAD Diagnostic Kit (Abbott Laboratories, North Chicago, IL). For the T_3 assay, nonradioactive T_3 standards were prepared in T_3-depleted rat serum and were substituted for the standards provided by the manufacturer. Rat serum was depleted of T_4 and T_3 by treatment with dextran-coated charcoal as previously described (27). The RIA for T_4 has previously been validated for rat serum (27). GH was measured by RIA according to the methods provided by the National Hormone and Pituitary Program, National Institutes of Health. The intra- and inter-assay coefficients of variation for all assays was < 10%.

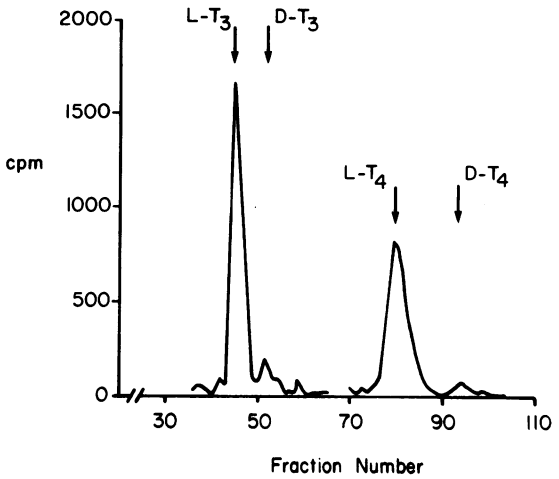

Figure 1. HPLC analysis of L-[^{125}I]T_3 and L-[^{125}I]T_4. Between 5,000–10,000 cpm of L-[^{125}I]T_3 and L-[^{125}I]T_4 together with 2.5 ng of L-T_3, L-T_4, D-T_3, and D-T_4 were injected into the HPLC column. The flow rate was 1.5 ml/min and 0.3-ml factions were collected.

Free T_4 and free T_3 fractions were determined in undiluted rat serum by an ultrafiltration method previously described (22). Free T_4 and free T_3 concentration in rat serum was then calculated as the product of the T_3 and T_4 concentrations and their respective free fractions.

Cell culture. Asynchronous monolayer cultures of GC cells were maintained in DME supplemented with 10% normal serum (calf/horse, 2:1; NS-DME) as previously described (28). Medium that was depleted of iodothyronines was prepared as follows: calf serum was treated with Dowex 1×8, 400-mesh, anion exchange resin according to the procedure described by Samuels et al. (23). This treatment was shown to decrease serum T_4 (Clinical Assays Kit, Travenol Laboratories, Inc.) from 58.0 to 1.3 nM and serum T_3 from 3.0 to < 0.05 nM (29). DME containing 10% resin-treated serum alone (RT-DME) or RT-DME that was supplemented with T_3 and T_4 were employed in various experiments as described below. The free T_3 and free T_4 fractions in RT-DME that was supplemented with various concentrations of T_3 and T_4 were also measured by the ultrafiltration method (22). The concentrations of free T_3 and free T_4 in RT-DME that was supplemented with T_3 and T_4 were calculated from the product of T_3 and T_4 concentrations in the medium and the respective free fraction for each iodothyronine.

Occupancy of T_3 receptors by iodothyronines. GC cells were incubated in 6-cm diam culture dishes with 10 ml of RT-DME supplemented with T_4, T_3, [^{125}I]T_4, and [^{131}I]T_3. We used a medium-exchange protocol (30) to minimize the cellular uptake of T_3 generated from T_4 [$T_3(T_4)$] that was released into the medium. In this protocol, the medium was exchanged every 10 h with 10 ml per dish of fresh and identical medium that was devoid of $T_3(T_4)$, as previously described (30). We have shown that this procedure limits the contribution of $T_3(T_4)$ to < 16% of the total medium T_3 (30).

Initial studies were done to determine the time of equilibration between nuclear and medium iodothyronines. In subsequent studies, cells were harvested after 24 h of incubation, a time when equilibrium between medium iodothyronines and cellular pools had been attained (See Results). The nuclear fraction was prepared (30) and assayed for radioactivity in an Autogamma Spectrometer (Packard Instruments Co., Downers Grove, Il). The counts per minute in the ^{125}I channel was

corrected for counts per minute of ^{131}I that appeared in the ^{125}I channel. Nuclear iodothyronines were then extracted by addition of 0.5 ml of 85% ethanol for 16 h at 20°C. < 5% of the ^{125}I or ^{131}I remained in the nuclear pellet after this procedure. Nuclear extracts were evaporated under a stream of N_2, resuspended in 50–100 μl of methanol/NH_3 (95:5, vol/vol). Samples were applied to Whatman 3 paper for descending chromatography in *t*-amyl alcohol/NH_3/hexane (5:6:1, vol/vol/vol) at 37°C as previously described (22). After chromatography, the paper was dried and the location of iodide, T_4, and T_3 was identified (22). The counts per minute corresponding to each component was then determined as described (22). Moles of $T_3(T_4)$ in individual nuclear samples was calculated from the product of the total counts per minute of ^{125}I, the percentage of total counts per minute corresponding to $[^{125}I]T_3$, determined by chromatography, and the specific activity of medium T_4 in each experiment. The result was then multiplied by 2 since only one half of the T_3 molecules generated from $[^{125}I]T_4$ are radiolabeled (31). The moles of exchangeable T_3 $[T_3(T_3)]$ and the moles of T_4 in a specific nuclear fraction were determined after paper chromatography as described above. The nonspecific binding of $T_3(T_3)$, $T_3(T_4)$, and T_4 in the nuclear fraction was measured by incubating the cells with 3,000 nM T_3. Nonspecific binding was always < 5% of the nuclear counts for $T_3(T_3)$, $T_3(T_4)$, and T_4. Nonspecific conversion of T_4 to T_3 was measured by incubating medium without cells at 37°C. Nonspecific conversion was < 0.05% of the counts added to the medium.

Interaction of T_3 and T_4 with T_3 nuclear receptors. T_3 nuclear receptor binding capacity was determined in each experiment. Dishes of GC cells were incubated for 24 h with different T_3 and T_4 concentrations according to the medium exchange protocol (30). After 24 h, cells were rinsed with serum-free medium and then incubated for 3 h with 5 nM $[^{125}I]T_3$ at 37°C. Under these conditions, > 90% of T_3 receptors are occupied with T_3. Cells were then harvested, and nuclei were isolated and assayed for radioactivity. Nonspecific binding was always < 5% of total binding.

TR affinity for T_3 and T_4 in GC cells nuclear extracts. Nuclear extracts were prepared as previously described (26). 200 μl of nuclear extract supplemented with 10 pM $[^{125}I]T_3$ and 0–5 nM nonradioactive T_3 or 0.2–500 nM nonradioactive T_4 in a total volume of 0.5 ml of 20 mM Tris/HCl, pH 7.8, 0.4M NaCl, and 5 mM dithiothreitol were incubated for 72 h at 0°C. Nonspecific binding, < 10% of total binding, was determined as previously described (32). At the end of incubation, receptor-bound $[^{125}I]T_3$ was separated from free $[^{125}I]T_3$ with Dowex 1×8, 400-mesh anion exchange resin as described (32). K_a of T_3 was calculated by Scatchard analysis. K_a of T_4 was calculated as the product of the molar ratio: $[T_3]/[T_4]$ that was observed to cause 50% displacement of $[^{125}I]T_3$ from TR (R), and the K_a of T_3. Thus, $K_aT_4 = K_aT_3 \cdot R$.

TR affinity for T_3 and T_4 in intact GC cells. GC cells were plated in NS-DME. At the beginning of each experiment, they were rinsed with serum-free medium and incubated for 4 h at 37°C with serum-free medium supplemented with 13 pM $[^{125}I]T_3$, 10 μM iopanoic acid, and 0–3 nM nonradioactive T_3 or 0–50 nM nonradioactive T_4. Cells were then harvested as described previously (33). Nuclear $[^{125}I]T_3$ and nonspecific binding were determined as previously described (33). The relative affinity of TR for T_3 and T_4 was assessed from the molar ratio $[T_3]/[T_4]$ that was observed to cause 50% displacement of $[^{125}I]T_3$ from TR during these incubations.

Data were expressed as mean±SEM. Statistical analyses were by Student's *t* test or analysis of variance when appropriate (34).

Results

Medium free T_4 and T_3 concentrations. Since GC cells were originally cloned from a rat GH-producing pituitary tumor, the relative roles of T_3 and T_4 in TR occupancy for the euthyroid state should be determined in incubations with physiological free T_3 and free T_4 concentrations. Initial studies were, therefore, designed to measure the free T_3 and free T_4 concentration of rat serum so that these concentrations could be duplicated in tissue culture medium (Table I). The intensity of rat serum protein binding of T_4 and T_3, was determined by ultrafiltration of undiluted serum. The bound/free ratio was 3,845 for T_4 and 120 for T_3. Thus, the intensity of T_4 binding by rat serum proteins was 32-fold greater than that of T_3. Mean rat serum free T_3 and free T_4 concentration were 8.8±2.2 and 15.4±1.7 pM, respectively ($P < 0.001$).

Table I. Total and Free T_3 and T_4 Concentrations in Rat Serum

	Total concentration	Free fraction	Free concentration
	nM	%	*pM*
T_3	1.07±0.07	0.83±0.04	8.8±0.7
T_4	60.9±2.8	0.026±0.001	15.4±0.6

Serum was collected from normal rats. Serum T_3 and T_4 were measured by radioimmunoassay and the free hormone fractions were measured by ultrafiltration of undiluted serum. Entries are mean±SE ($n = 9$).

The concentration of T_3 and T_4 in RT-DME that would result in free T_3 and free T_4 concentrations similar to physiological free T_3 and free T_4 concentrations of rat serum was determined by measuring free T_3 and free T_4 in RT-DME that was enriched with increasing concentrations of T_3 and T_4 (Fig. 2). In this experiment, supplementation of RT-DME with 110 pM T_3 and 1.4 nM T_4 resulted in medium free T_3 (MFT_3) and medium free T_4 (MFT_4) concentrations that were the same as the respective free hormone concentrations in rat serum. During these studies, we noted some variability in the concentration of medium T_3 and T_4 that were necessary to establish physiological conditions. Further experiments revealed that treatment of calf serum with resin to deplete serum of thyroid hormones resulted in a variable decrease in binding of T_4 and T_3 (Table II). Because of this variability, unless otherwise indicated, experimental media used in these studies were studied as in Fig. 2 in order to be certain that MFT_3 and MFT_4 were similar to those in rat serum.

Occupancy of nuclear receptors by T_4. Initial studies were done to determine the time at which equilibrium had occurred between nuclear and medium iodothyronines in incubations of GC cells employing the medium exchange protocol (Table III). Receptor occupancy by $T_3(T_3)$, $T_3(T_4)$, and T_4 achieved after 22 h of incubation was equivalent to that achieved after 28 h of incubation. These findings suggested that medium iodothyronines were equilibrated with nuclear iodothyronines after 22 h of incubation. All subsequent studies were therefore carried out after 24 h of incubation.

The occupancy of nuclear TR by T_4 was studied in GC cells cultured with near-physiological MFT_3, achieved by supplementing RT-DME with 85 pM T_3, and with different concentrations of medium T_4, 0.5–5.0 nM. These medium T_4 concentrations were selected because they were both below and above 1.4 nM, the concentration that results in physiological free T_4, (15.4 pM). As seen in Fig. 3, which depicts a representative experiment, incubation with increasing medium T_4 concentrations resulted in a progressive and significant increase in TR

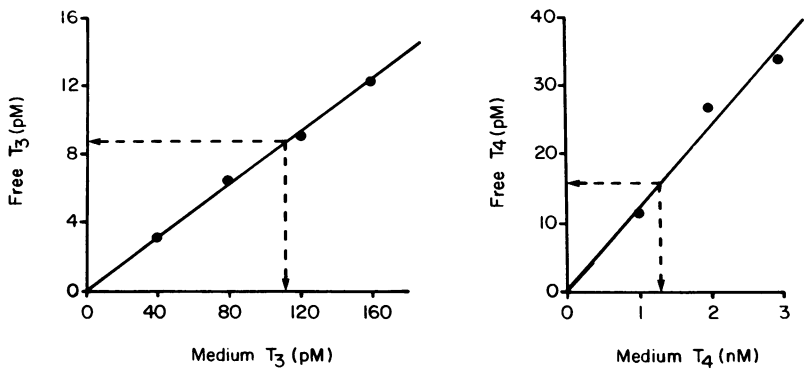

Figure 2. Concentration-dependence of free T_3 and free T_4 on total medium T_3 and T_4 concentrations in 10% RT-DME. 10% RT-DME was supplemented with T_3 (*left*) or T_4 (*right*). Free hormone fractions were determined by ultrafiltration. The arrows on the ordinate represent the specific free T_3 and free T_4 concentrations that occur in euthyroid rat serum (Table I).

occupancy by both T_4 and $T_3(T_4)$ as well a decrease in TR occupancy by $T_3(T_3)$. These iodothyronines together occupied 28.8±0.1% of TR binding sites when GC cells were incubated with 0.5 nM T_4. TR occupancy by all iodothyronines increased progressively with increasing medium T_4 to 41.0±1.0% occupancy when GC cells were incubated with 5.0 nM T_4. At physiological MFT_4, 15.4 pM, T_4 accounted for 7.5%, $T_3(T_4)$ for 32.5%, and $T_3(T_3)$ for 60.0% of the occupied TR binding sites. After incubation with 5.0 nM T_4, which was assumed to increase MFT_4 3.6-fold, T_4 accounted for 20.4%, $T_3(T_4)$ for 40.3%, and $T_3(T_3)$ for 39.3% of the occupied TR binding sites. Thus, T_4 and T_3 derived from T_4 constituted the major portion of iodothyronine bound to TR in GC cells cultured with raised concentrations of MFT_4.

Estimation of nuclear free iodothyronine concentration. The magnitude of the contribution of T_4 to TR occupancy, illustrated in Fig. 3, was unanticipated since we had assumed that the T_3 and T_4 concentrations in nucleus would change in proportion to the MFT_3 and MFT_4 concentrations. This is because our studies (see below) suggested that the affinity of TR for T_4 was < 3% of the affinity of T_3. Since FT_3 concentration in the nucleus (NFT_3) has been reported to be greater than that in plasma (35), the present findings might be explained by nuclear free T_4 (NFT_4) concentrations that were disproportionately greater than those of NFT_3. This hypothesis was tested by calculating the nuclear free iodothyronine concentration [H] from the law of mass action (35): $K_a = [TR_O]/([H][TR_u])$.

The concentration of occupied TR, $[TR_O]$, and unoccupied TR, $[TR_u]$, can readily be determined experimentally as in Fig. 3. K_a for T_3, K_aT_3, was determined by Scatchard analysis in nuclear extracts of GC cells (Fig. 4). In three experiments, K_aT_3 was 6.3×10^9, 8.6×10^9, and 6.1×10^9 liters/mol; mean K_aT_3 was $7.0 \pm 1.4 \times 10^9$ liters/mol.

Three experiments to determine nuclear occupancy by T_4 and $T_3(T_3)$ were carried out in GC cells incubated at physiological MFT_3 and MFT_4 and the results were used to calculate NFT_3 (Table IV). Mean NFT_3 varied between 29.4±0.2 and 50.0±3.1 pM in the three experiments, and the nuclear/medium ratio (N/M) for free T_3 varied between 3.4±0.3 and 5.9±0.4. These findings indicate that free T_3 is concentrated in the nucleus in comparison to the medium and are concordant with data reported for rat liver, kidney, brain, and heart (35).

The relative displacement of $[^{125}I]T_3$ by T_3 and T_4 in the nuclear extracts of GC cells is illustrated in Fig. 5. For three experiments, the concentration of T_4 required for 50% displacement of $[^{125}I]T_3$ was 42-, 40-, and 44-fold greater than the concentration of T_3 required for 50% displacement of $[^{125}I]T_3$. Thus, mean $R = 1/42 \pm 1.2 = 0.024 \pm 0.006$ and $K_aT_4 = K_aT_3 \cdot R = 0.17 \pm 0.04 \times 10^9$ liters/mol.

The NFT_4 concentration was calculated using the calculated K_aT_4 and the TR occupancy data from three experiments carried out at physiological MFT_4 and MFT_3 concentration

Table II. Effect of Resin Treatment of Calf Serum on the Free Fraction of T_3 and T_4

	Free hormone fraction		
	Control	Resin treatment	*P*
	%	%	
T_3	0.455±0.094	0.841±0.126	<0.025
T_4	0.043	0.100	

Resin-treated calf serum was prepared as in Methods. T_3 and T_4 were added to resin-treated calf serum to restore the concentrations that were present before treatment with resin. Entries for T_3 are mean±SE (n = 3). Entries for T_4 are the average of duplicate measurements which varied < 5% from the average value.

Table III. Equilibration of Nuclear and Medium Thyroid Hormones

	T_3 nuclear receptors			
		Occupancy		
Duration of incubation	Total	T_4	$T_3(T_4)$	$T_3(T_3)$
h	*fmol/dish*	%	%	%
22	356.3±17.0	8.1±0.7	11.6±0.6	5.9±0.3
28	372.7±14.4*	8.4±0.3*	12.7±0.3*	6.2±0.3*

GC cells were plated in NS-DMEM. After 3 d they were rinsed with RT-DME and then incubated with RT-DME supplemented with 40 pM T_3 and 2.5 nM T_4. The medium exchange protocol described in Methods was used to minimize the cellular reuptake of $T_3(T_4)$. Nuclear TR occupancy was determined after 22 and 28 h as per Methods. Each value represents the mean±SE for three determinations.

* Not significantly different, $P > 0.05$.

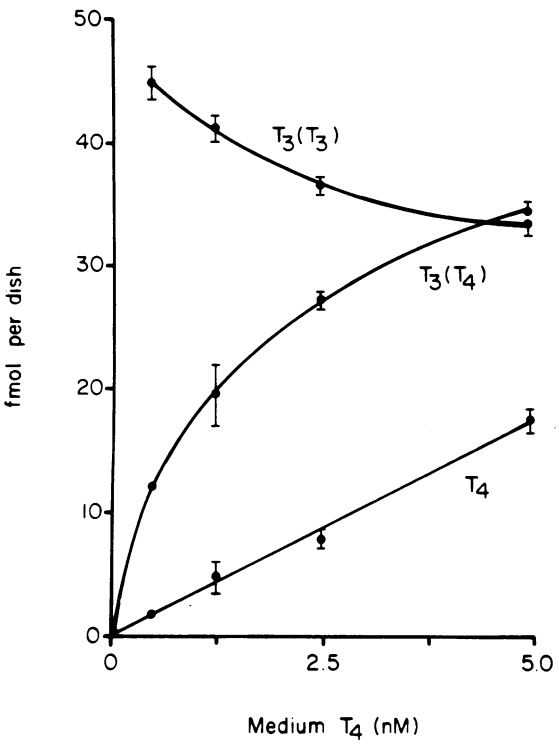

Figure 3. Effect of increasing medium T_4 on T_3 nuclear receptor occupancy. GC cells were plated in NS-DME for 48 h. Cells were rinsed briefly with RT-DME and then cultured in RT-DME supplemented with 85 pM T_3 and increasing concentrations of T_4 (0.5–5.0 nM). The medium exchange procedure (see Methods) was employed for 24 h to minimize accumulation and cellular reuptake of $T_3(T_4)$. Cells were then harvested and nuclear TR occupancy determined as described in Methods. Entries are mean±SE (n = 3).

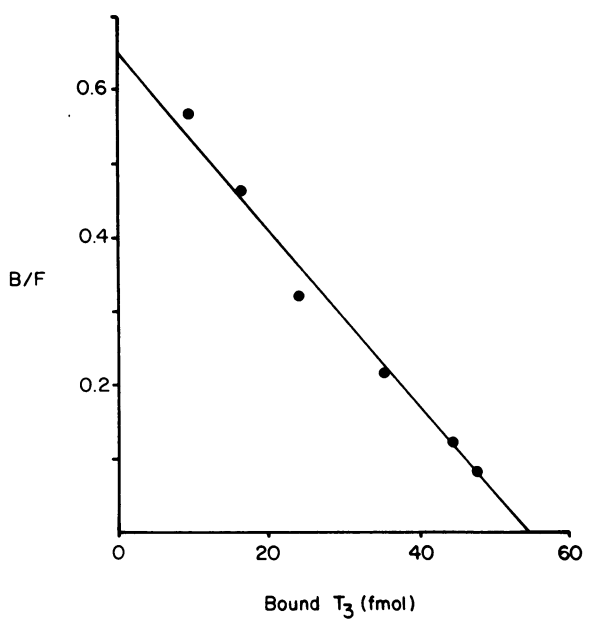

Figure 4. Scatchard analysis of T_3 binding to T_3 receptors in nuclear extracts. GC cells were cultured with NS-DME for 48 h. Cells were harvested and nuclear extracts prepared as described in Methods. Each point is the average of two measurements which differed < 10%.

(Table IV). Mean NFT_4 was 12.4±0.7-, 19.3±2.3-, and 6.5±0.3-fold greater than MFT_4 concentration in three experiments. Moreover, mean NFT_4 concentration significantly exceeded mean NFT_3 concentration in all studies. Further analysis of the N/M ratios of free T_4 and free T_3 revealed that mean N/M free T_4 was 2.7±0.1-, 3.3±0.2-, and 1.9±0.1-fold greater than N/M free T_3 in the three experiments. These findings suggest that, relative to medium free iodothyronine concentration, free T_4 is concentrated in the nucleus to an ~ 2.6-fold greater extent than free T_3 during incubations at physiological MFT_3 and MFT_4.

Since free T_4 is concentrated in the nucleus to a greater extent than free T_3, as suggested from our studies employing nuclear extracts, the concentration of T_4 in medium relative to T_3 that would be necessary to displace T_3 from nuclear TR in intact cells should be significantly smaller than that observed in nuclear extracts (T_4 42.0±1.2-fold > T_3). The displacement of [^{125}I]T_3 from TR by T_3 and T_4 was, therefore, studied in GC cells which were incubated with 10 μM iopanoic acid to inhibit T_3 production from T_4. Preliminary experiments showed that incubation with 10 μM iopanoic acid decreased nuclear $T_3(T_4)$ to less than 3% of that found in control cultures incubated in the absence of iopanoic acid. The maximal amount of T_3 generated from T_4 under these experimental conditions was insufficient to displace [^{125}I]T_3 from TR. Secondly, studies using nuclear extracts revealed that iopanoic acid did not influence the interaction of either T_3 or T_4 with TR. Lastly, studies with intact GC cells showed that iopanoic acid did not alter the concentration of $T_3(T_3)$ or T_4 in the nucleus.

A representative study of T_4 and T_3 interaction with TR in intact cells, shown in Fig. 6, indicates that the concentration of T_4 required for 50% displacement of [^{125}I]T_3 from nuclear TR was 8.3-fold greater than the concentration of T_3 required for 50% displacement of [^{125}I]T_3. The mean value was 9.1±1.5-fold (n = 3), significantly smaller than observed in studies employing nuclear extracts, 42.0±1.2-fold ($P < 0.001$).

Relationship between medium T_4 and NFT_4. We used data from the experiment shown in Fig. 3 and the apparent equilibrium association constant for T_3 and T_4, listed above, to determine whether changes in medium T_4 concentration would be reflected by comparable changes in NFT_4 (Table V). GC cells were incubated with 0.085 nM T_3, which resulted in near-physiological MFT_3. NFT_3 was about five-fold greater than MFT_3 and was not influenced by increasing medium T_4, over a wide range. Increasing medium T_4 from 0.5 to 5.0 nM resulted in parallel changes in NFT_4. NFT_4 always exceeded NFT_3. At physiological MFT_4, observed in incubations with 1.25 nM T_4, NFT_4 was 4.9-fold greater than NFT_3.

T_4 occupancy of nuclear TR when medium T_3 is decreased. Two experiments were carried out to determine the occupancy of TR by T_4 and $T_3(T_4)$ when medium T_3 was decreased, an experimental protocol that has relevance to sick patients with decreased serum free T_3 (Table VI). The total number of TR was not influenced by changes in medium T_4 and T_3 in these experiments. However, a decrease in medium T_3 from physiological levels, 0.11 nM, to 0.03 nM resulted in a significant decrease in abundance of total occupied TR (Table VI, B). TR occupied by $T_3(T_3)$ decreased and TR occupied by $T_3(T_4)$ increased significantly even in the absence of changes of medium T_4. When medium T_4 was increased from 1.4 to 2.8 nM, in the continuing presence of decreased medium T_3, TR total occu-

Table IV. Relationship between Nuclear and Medium Free Iodothyronine Concentrations after Incubation of GC Cells with Physiological MFT_4 and MFT_3

Experiment number	Nuclear free iodothyronine		Nuclear/medium (N/M) free iodothyronine ratios		Free T_4 N/M / Free T_3 N/M
	T_4	T_3	T_4	T_3	
	pM	*pM*			
1	185	41.1	13.7	4.8	2.9
	164	39.6	12.1	4.7	2.6
	153	36.9	11.3	4.3	2.6
	167±9.4	39.2±1.2	12.4±0.7	4.6±0.2	2.7±0.1
P<	0.001		0.001		
2	232	43.9	15.0	5.2	2.9
	313	53.7	20.2	6.3	3.2
	353	52.5	22.8	6.2	3.7
	299±35.6	50.0±3.1	19.3±2.3	5.9±0.4	3.3±0.2
P<	0.005		0.005		
3	109	29.2	7.0	3.4	2.1
	95	29.2	6.1	3.4	1.8
	97	29.7	6.3	3.5	1.8
	100±4.4	29.4±0.2	6.5±0.3	3.4±0.3	1.9±0.1
P<	0.001		0.001		

Nuclear free iodothyronine concentrations were calculated as described in Results using the mass action equation. Medium free iodothyronine concentration was determined from the product of the iodothyronine concentration and the free iodothyronine fraction which was measured by ultrafiltration. Entries are mean±SE (n = 3).

pancy was restored to or near control values (Table VI, C). TR receptor occupancy was restored by a significant increase in occupancy by both T_4 and $T_3(T_4)$.

The decrease in TR total occupancy observed when medium T_3 was decreased (Table VI, B) and the restoration of TR occupancy to or near control values when medium T_4 was increased (Table VI, C) were paralleled by significant changes in GH production.

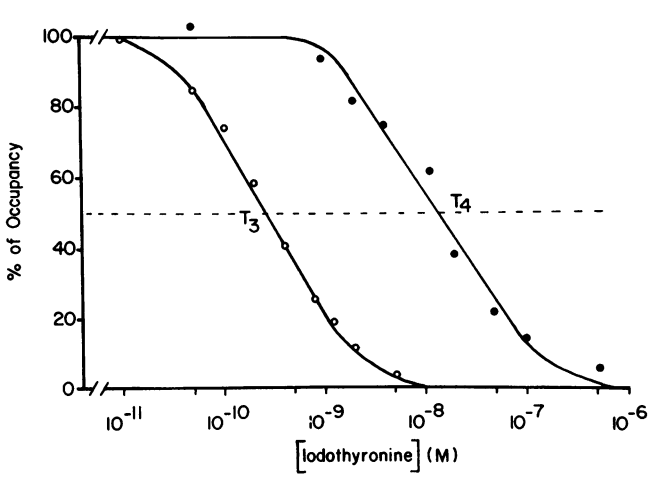

Figure 5. Relative displacement of $[^{125}I]T_3$ from nuclear thyroid receptors in nuclear extracts. Nuclear extracts were prepared as described in legend to Fig. 4. Each point is the average of two determinations which differed < 10%.

Discussion

Since GC cells were derived from a rat pituitary tumor, the premise of this investigation was that information potentially relevant to the intact euthyroid rat would most likely be ob-

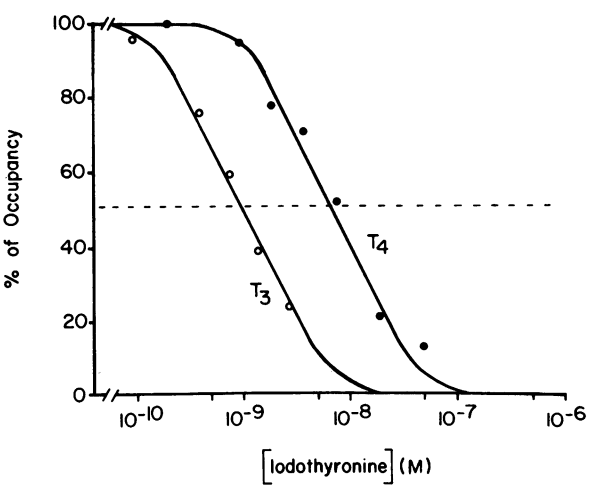

Figure 6. Relative displacement of $[^{125}I]T_3$ from nuclear receptors during incubation of GC cells with different concentrations of T_3 and T_4. GC cells were plated in NS-DME for 72 h. Cells were rinsed with serum free medium and then incubated with serum free medium supplemented with 13 pM $[^{125}I]T_3$, 10 μM iopanoic acid and increasing concentrations of either T_3 or T_4. Cells were harvested after 4 h for determination of $[^{125}I]T_3$ specifically bound to nuclei. Each point is the average of duplicate measurements which varied < 10%.

Table V. Relationship between Medium T_4 and Nuclear Free Iodothyronine Concentration

Medium		Nuclear free iodothyronine		$\frac{NFT_4}{NFT_3}$
T_4	T_3	T_4	T_3	
nM	*nM*	*pM*	*pM*	
0.5	0.085	76.6±1.4	44.2±0.2	1.7
1.25	0.085	216.7±22.4	44.3±1.8	4.9
2.5	0.085	363.1±17.5	41.5±0.8	8.7
5.0	0.085	895.1±84.2	42.0±3.4	21.3

Nuclear free iodothyronine concentrations were calculated as described in Results using the mass action equation. Nuclear occupancy data were from the experiment depicted in Fig. 2. Entries are mean±SE (n = 3).

tained when cells were exposed to medium free iodothyronine concentrations similar to those in euthyroid rat serum. Physiological medium free iodothyronine concentrations were achieved by supplementing RT-DME with between 90 and 115 pM T_3 and 1.2–1.4 nM T_4, concentrations that are significantly less than those reported previously to restore physiological concentrations (28). This difference results from the new finding that treatment of calf serum with Dowex 1×8 anion exchange resin to deplete endogenous iodothyronines increases the free fraction of both T_4 and T_3. The present experiments, however, do not define whether this increase in free fraction results from depletion of iodothyronine binding proteins or other constituents of serum that facilitate binding of iodothyronines.

After incubation of GC cells with physiological MFT_4 and MFT_3 concentrations, T_4 accounted for 5.4–10.0% of the occupied nuclear TR sites, a finding that is concordant with a previous reports concerning liver and kidney of euthyroid rats (15). Exposure of cells to increasing MFT_4 in the presence of near physiological MFT_3 concentration resulted in a progressive increase in the contribution of T_4 and $T_3(T_4)$ to nuclear TR occupancy and a modest decrease in occupancy by $T_3(T_3)$. When GC cells were maintained in MFT_4 concentrations that were fourfold greater than the euthyroid value, T_4 itself constituted 20% of the iodothyronine bound to TR. Lastly, incubation with increasing MFT_4 was associated with an increase in total receptor occupancy from 20–30% in cells maintained at physiological MFT_4 to 35–45% in cells cultured with fourfold increased concentrations of MFT_4. Thus, in addition to an important role as a prohormone for $T_3(T_4)$ in GC cells, the present studies establish a role for T_4 itself in thyroid hormone action in GC cells cultured with physiologic concentrations of MFT_4 and MFT_3 and a much larger role when MFT_4 is selectively increased.

A common clinical setting that is associated with altered serum free iodothyronine concentrations is the euthyroid patient with acute nonthyroidal disease. Such patients are often characterized by decreased concentrations of serum free T_3 and either unchanged or increased concentrations of serum free T_4 (20–22, 36). A role for the normal or raised serum free T_4 concentration in the maintenance of the euthyroid state in these patients has not been established. The present experiments suggest that T_4 may play an important role in the maintenance of normal TR occupancy and biologic activity when MFT_3 is decreased. A decrease in MFT_3 without changing MFT_4 was associated with a proportional decrease in $T_3(T_3)$ bound to nuclear TR, partially offset by a significant increase

Table VI. Effect of Changes in Medium T_4 and T_3 Concentrations on T_3 Nuclear Receptor Occupancy and GH Production

Medium iodothyronine		Incubation condition	T_3 nuclear receptors					Medium GH
				Occupancy				
T_3	T_4		Total	T_4	$T_3(T_4)$	$T_3(T_3)$	Total	
nM			*fmol/dish*	%	%	%	%	*ng/dish*
0.11	1.4	A	397.3±2.1	1.3±0.1	6.8±0.2	15.7±0.1	23.8±0.2	5,371±100
0.03	1.4	B	390.6±7.1	2.0±0.1	10.2±0.2	6.4±0.3	18.7±0.7	3,436±59
0.03	2.8	C	395.7±43.9	5.1±0.9	12.4±1.1	6.2±0.4	23.7±2.41	4,423±64
		A vs. B	NS	NS	<0.001	<0.001	<0.1 > 0.05	<0.001
		A vs. C	NS	<0.005	<0.005	<0.001	NS	<0.001
		B vs. C	NS	<0.005	<0.025	NS	<0.1 > 0.05	<0.01
0.11	1.4	A	393.5±11.5	3.4±0.3	7.3±0.4	23.1±0.9	33.8±1.6	4,790*
0.03	1.4	B	380.3±7.4	3.9±0.1	11.2±0.3	8.0±0.2	23.1±0.3	3,758±148
0.03	2.8	C	377.0±8.1	8.1±0.1	12.1±0.2	7.4±0.1	27.7±0.3	4,907±192
		A vs. B	NS	<0.05	<0.001	<0.001	<0.001	<0.01
		A vs. C	NS	<0.001	<0.001	<0.001	<0.005	NS
		B vs. C	NS	<0.001	$P > 0.1 < 0.05$	NS	<0.01	<0.005

GC cells were plated in NS-DME for 24 h. The cells were rinsed with serum-free medium and exposed to RT-DME supplemented with iodothyronine concentrations listed above. The medium-exchange procedure described in Methods was used to minimize the accumulation and cellular reuptake of $T_3(T_4)$. Nuclear TR occupancy was determined after 24 h as per Methods and, in separate dishes, medium was collected between 24 and 48 h for measurement of GH by RIA. Each value represents the mean±SE for three determinations. * Average of duplicate measurements that varied < 10%.

in TR-associated $T_3(T_4)$. Total nuclear TR occupancy by iodothyronines declined and this was paralleled by a decrease in GH production. The increase in $T_3(T_4)$ bound to TR under these conditions could be due to development of cellular hypothyroidism which has been associated with increased activity of type II T_4-5′ deiodinase (37). Alternatively, the finding could be due to a decrease in product, (T_3), inhibition of the enzyme. Published reports suggest the former possibility is more likely since T_3 appears to have only minimal affect on enzyme activity (38).

When MFT_4 concentration was increased to twofold the physiological value, nuclear TR occupancy and GH production were restored to or near the value observed when both MFT_4 and MFT_3 concentrations were similar to those in euthyroid rat serum. Both T_4 and a further increase in $T_3(T_4)$ contributed nearly equally to the restoration of nuclear TR occupancy under these conditions. These findings indicate that increased MFT_4 concentrations significantly affected TR occupancy and biologic activity in GC cells that were incubated with decreased MFT_3. A similar role may be postulated for the raised serum free T_4 concentration that occurs in many patients with nonthyroidal disease.

The significant contribution of T_4 to nuclear TR occupancy in the present studies prompted a reexamination of TR interactions with T_4 and T_3. Studies of the relative concentrations of T_4 and T_3 that were required for 50% displacement of T_3 from TR sites in nuclear extracts of GC cells revealed that the avidity of TR for T_4 was only 2.4% that of T_3. In the present studies, the avidity of nuclear sites for T_4, 2–4% that of T_3, was significantly less than reported for isolated rat liver nuclei, 8% that of T_3 (39, 40), nuclei from cultured GH_1 cells, 10% that of T_3 (39), or for extracts of rat liver nuclei or GH_1 cell nuclei, 10% that of T_3 (41). Nuclear TR receptors have recently been shown to be the cellular homologues of c-erb A proto-oncogenes (42) and several forms of TR that bind T_3, $\alpha 1$, $\beta 1$, and $\beta 2$, occur in different tissues. A number of these receptor forms from different tissues have been generated in vitro for studies of the binding of T_3, T_4, and iodothyronine analogues. A review of these data revealed a wide variation in the apparent avidity of TR for T_4 relative to T_3 that was not segregated to specific receptor forms. For α TR forms, the T_4 interaction relative to T_3 was 3.7% for TR from rat brain (43), 13.3% for TR from chicken embryonic tissue (44), 2.3% for TR from human kidney (45), and 11.5% for TR from rat liver (46). For β TR forms, the TR interaction relative to T_3 was 17.9% for TR from human placenta (47) and 5% for TR from rat liver (46). The wide variation in apparent avidity of different TR forms for T_4 relative to T_3 suggests that significant differences in reaction conditions prevailed in these published reports. The T_3 content of T_4 solutions was not mentioned in any of these reports. In our view, variable T_3 contamination of T_4 solutions that were used to displace T_3 is a likely explanation for the differences between these studies. If the intrinsic avidity of TR sites for T_4 is 2–3% that of T_3, as in the present report and in the report of Nakai et al. (45), 1–2% contamination of T_4 solutions with T_3 would result in a significant increase in the apparent avidity of receptor for T_4. The magnitude of the affect of T_3 contamination would also be dependent of the degree of saturation of TR sites with radioactively labeled T_3.

Using the apparent association constants of TR for T_3 and T_4, described herein, the calculated NFT_3 was determined to be significantly greater than MFT_3. This finding extends the observations of Oppenheimer and Schwartz (35) to cultured GC cells. The raised concentration of NFT_3 relative to MFT_3 (3–6-fold) in cultured GC cells is appreciably smaller than reported for NFT_3 in nuclei from rat liver, kidney, heart and brain (range: 50–250-fold) (35) but similar to the 7.6-fold gradient reported by Freake et al. (25) in cultured GH_1 cells. The present studies also suggest that free T_4 is concentrated in GC cell nuclei (6–20-fold greater than MFT_4) and to a greater extent than T_3. Relative to MFT_4 and MFT_3 concentration, NFT_4 was concentrated to 1.9–3.3-fold greater extent than NFT_3. The conclusion that NFT_4 was concentrated in nuclei to a greater extent than NFT_3 was strengthened by data obtained in intact GC cells maintained in 10 μM iopanoic acid. The iopanoic acid decreased T_3 production from T_4 to < 3% of control values and neither influenced the avidity of TR for T_3 or T_4 nor the delivery of T_4 into the cell. The results of these studies indicated that significantly smaller concentrations of T_4 relative to T_3 were required to displace T_3 from TR sites in intact cells than in nuclear extracts. A likely explanation for the difference is that free T_4 is concentrated to a greater extent than free T_3 in intact cells.

The present results do not provide new information concerning the cellular mechanisms responsible for maintaining the increased NFT_4 and NFT_3 gradients. Oppenheimer and Schwartz (35) reported that the principle gradient for free T_3 occurred between cytosol and nuclear compartments rather than between the plasma and cytosol compartments. They also suggested that the free T_3 gradient was probably maintained by an energy-dependent process (35). The finding that free T_4 gradients occur between nuclei and medium free T_4 in cultured GC cells and that the free T_4 gradients are larger than comparable gradients for free T_3 raise the possibility that separate transporters may exist for the two iodothyronines or that the affinity of a common transporter for T_4 is larger than for T_3.

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