

Comparative Study of Pituitary–Thyroid Hormone Economy in Fasting and Hypothyroid Rats

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Abstract

Starvation in laboratory rodents results in significant alterations in thyroid hormone economy characterized by decreased circulating levels of thyroxine (T_4) and 3,5,3'-triiodothyronine (T_3) and a decline in serum thyrotropin (TSH) concentration. To investigate this apparent paradox, we have compared in fasted and hypothyroid animals the intracellular parameters mediating thyroid hormone action in the anterior pituitary gland. In vitro saturation analysis combined with quantitation of nuclear T_3 content by radioimmunoassay allowed for characterization of pituitary nuclear T_3 receptors and estimation of the endogenous fractional receptor occupancy. In rats, thyroidectomized 4 wk earlier, the 10-fold increase in serum TSH levels and decline in peripheral thyroid hormone concentrations were accompanied by a 61% decrease in pituitary nuclear T_3 content and a marked decline in fractional T_3 receptor occupancy as compared with control animals. In euthyroid animals subjected to short-term starvation (72 h), serum T_3 , T_4 , and TSH levels declined by 52, 43, and 48%, respectively. Despite these marked decreases in circulating thyroid hormone levels, pituitary nuclear T_3 content in fasted rats declined by only 15% ($P < 0.05$) relative to control levels. This modest decline in nuclear T_3 content, combined with a 23% decrease in total T_3 receptor number, resulted in an estimated fractional receptor occupancy in fasted animals which was equal to or greater than that noted in controls. The effects of fasting and hypothyroidism on the pituitary were further investigated by quantifying low Michaelis constant (K_m) T_4 5'-deiodinase activity in the crude cytosol fraction of pituitary homogenates. In thyroidectomized animals, maximum velocity was increased ninefold, whereas fasting resulted in a 37% decrease ($P < 0.025$) in this parameter compared with controls. K_m values were similar in all experimental groups (4.7 ± 0.6 nM).

These results demonstrate that, despite significant reductions in circulating thyroid hormone concentrations and pituitary T_4 5'-deiodinase activity, nuclear T_3 levels are maintained at relatively normal levels in the pituitary of the fasted animal and fractional T_3 receptor occupancy may actually increase. These findings are in marked contrast to those noted in thyroidectomized animals and suggest that the suppression of TSH secretion accompanying starvation in the rat is mediated, at least in part, by local pituitary mechanisms that serve to maintain and possibly enhance nuclear T_3 receptor occupancy.

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Introduction

In both humans and laboratory animals, nutritional status profoundly affects thyroid hormone economy and pituitary function (1–4). In humans, short-term starvation is accompanied by significant decreases in serum total and free 3,5,3'-triiodothyronine (T_3)¹ levels whereas thyroxine (T_4) levels are unaltered or slightly elevated (1, 3, 5, 6). In rodents, a 48–72-h fast results in marked depressions of both free and total T_3 and T_4 serum levels (7, 8). Evidence now suggests that these lowered peripheral thyroid hormone levels have a protein-sparing effect and thus may be of considerable adaptive value (6, 9–12).

The hypothalamic–pituitary axis appears to play an important role in maintaining the apparent thyroid hormone-deficient status of fasting animals. Thus, despite decreased peripheral thyroid hormone levels, serum thyrotropin (TSH) levels do not rise (1, 3, 5, 6). Indeed, in the rat, inanition results in decreased serum and intrapituitary TSH levels and eventual thyroid gland atrophy (2, 7, 13). The pituitary, however, remains sensitive to thyroid hormone feedback (6, 14, 15) and the diurnal rhythm of TSH secretion is unchanged (16) suggesting an altered “set-point” for TSH regulation in the starving animal.

The physiologic mechanisms responsible for these adaptive changes in pituitary function are uncertain, but they could result from alterations in the secretion of hypothalamic hormones or be mediated by direct effects of nutritional deprivation as noted in the liver (17). Alternatively, fasting could induce changes in thyroid hormone economy in the pituitary. Only limited data are available, however, concerning the effects of starvation on the pituitary cellular mechanisms that influence thyroid hormone action. Based on the results of a single experiment using in vivo saturation techniques, Bavli (18) has suggested that pituitary nuclear T_3 receptor levels are decreased in fasting rats. Others have reported that pituitary T_4 5'-deiodinase activity is unchanged (19, 20) or decreased (21, 22) with starvation. How these alterations relate to fasting-induced changes in pituitary function is uncertain.

In the present study, we have utilized in vitro techniques to examine and compare in the rat the effects of short-term starvation and hypothyroidism on pituitary nuclear T_3 receptor levels, nuclear T_3 content, and the kinetics of T_4 5'-deiodination. Our results demonstrate marked differences in pituitary–thyroid hormone economy in these two conditions. These differences may have important implications in the control of pituitary function and TSH secretion.

1. Abbreviations used in this paper: DTT, dithiothreitol; MBC, maximum binding capacity; NSB, nonspecific binding; T_3 , 3,5,3'-triiodothyronine; T_4 , thyroxine; TSH, thyrotropin.

Methods

Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA) and were kept under conditions of controlled lighting and temperature. Fasting experiments (protocol I) were conducted with 8-wk-old animals that had initial weights of 200–225 g. Before killing, animals were fasted for a 72-h period during which time they were allowed free access to water and housed individually in metal cages with wire-mesh floors to prevent coprophagia. Control animals were maintained in a similar manner but were allowed free access to pelleted rat diet. For experiments involving hypothyroid and T_4 -injected animals (protocol II), normal and thyroidectomized rats (7 wk old, 175–200 g in initial body wt) were purchased from the same supplier. Animals were housed in pairs in clear plastic cages with wood shavings for bedding and were given free access to food and water. Thyroidectomized animals were given drinking water containing calcium lactate (0.9 g/dl). Animals were killed 4–5 wk postsurgery. T_4 -treated animals received 30 μ g of T_4 /100 g of body wt injected subcutaneously for 18 d before killing and were utilized 24 h after the last injection.

Tissue preparation. Animals were stunned and decapitated, and trunk blood was collected. Anterior pituitaries from 4 to 11 animals in each experimental group were pooled, weighed, and homogenized by hand in 30 vol (wt/vol) of ice-cold homogenate buffer (0.25 M sucrose, 0.02 M Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM dithiothreitol [DTT], 5% glycerol). Subsequent procedures were carried out at 0–4°C. The crude homogenate was centrifuged at 800 g for 10 min, and the supernate (designated cytosol hereafter for convenience) was used immediately for determination of T_4 5'-deiodinase activity as described below. The pellet was utilized to prepare a purified suspension of isolated nuclei (verified by Nomarski interference-contrast techniques) by modification of the method of Spindler et al. (23). The pellet was washed twice with 2.5 ml of homogenate buffer containing 0.5% Triton X-100, and then washed once and resuspended in incubation buffer (0.25 M sucrose, 0.02 M Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 0.1 mM DTT, 2 mM EDTA, 50 mM NaCl, 5% glycerol) using a Dounce glass homogenizer. Aliquots of the nuclear suspension were then utilized in T_3 saturation studies. ~60% of the DNA in the crude homogenate (as determined by the method of Burton [24]) was recovered in the purified nuclear preparation.

Characterization of nuclear T_3 binding sites. Aliquots of the nuclear suspension containing 15–35 μ g DNA were incubated in a total volume of 1 ml of incubation buffer containing 4×10^{-11} M ^{125}I - T_3 (sp act ~1,200 μ Ci/ μ g, New England Nuclear, Boston, MA) and stable T_3 (Sigma Chemical Co., St. Louis, MO) sufficient to yield total concentrations of 5×10^{-11} to 5×10^{-9} M. Nonspecific binding (NSB) of ^{125}I - T_3 was determined in all experiments by incubating nuclei with 1 or 5×10^{-7} M stable T_3 . Incubations were performed at 22°C or 37°C for varying periods of time. After incubation, nuclei were chilled to 0–4°C and centrifuged at 1,200 g for 10 min, and a 0.5-ml aliquot of supernate removed for determination of free hormone concentration by absorption on dextran-coated charcoal (25). Greater than 94% of the total hormone in the supernate was in the free form. The nuclear pellet was washed once with an ice-cold solution of 0.5% Triton X-100 (in 0.25 M sucrose, 0.02 M Tris-HCl, pH 7.6, 1 mM $MgCl_2$, 0.1 mM DTT, 5% glycerol), resuspended in 0.5 ml of homogenate buffer, and counted in an automatic gamma counter (model 1195, Amersham/Searle, Arlington Heights, IL). The suspension was then stored at –20°C for subsequent DNA analysis. The single Triton X-100 wash reduced NSB of ^{125}I - T_3 to <6% of total binding. Scatchard analysis (26) of the binding data was carried out graphically (27). Points represent single determinations.

Several experiments (each performed at least twice) were undertaken to determine the optimal conditions for saturation analysis. (a) The time course of in vitro association of T_3 to isolated pituitary nuclei was determined by incubating aliquots of nuclei from euthyroid animals with 10^{-10} M ^{125}I - T_3 for varying lengths of time and then quantitating the amount of bound ^{125}I - T_3 . (b) The rate of dissociation

after in vitro labeling was measured by adding 5×10^{-7} M stable T_3 to aliquots of nuclei previously incubated with 10^{-10} M ^{125}I - T_3 and determining the time course of disappearance of radioactivity from the nuclear pellet. (c) To determine the rate of in vitro dissociation of endogenously bound T_3 , euthyroid rats were injected intravenously with a “tracer” amount of ^{125}I - T_3 (4 ng/100 g body wt in phosphate-buffered saline containing 10% normal rat serum) 60 min before killing. Pituitary nuclei were then isolated and incubated with 10^{-6} M stable T_3 . At varying time intervals the nuclei were chilled to 0–4°C, and bound ^{125}I - T_3 was determined as described above. The rate of dissociation was estimated by determining the time course of both ^{125}I - T_3 disappearance from the nuclear pellet and ^{125}I - T_3 appearance in the incubation medium. NSB was assessed in a second group of euthyroid rats by the simultaneous injection of 20 μ g of stable T_3 /100 g of body wt with the ^{125}I - T_3 . NSB was 3% of total ^{125}I - T_3 binding. (d) To assess further the effects of incubation conditions on the saturation analyses, euthyroid rats were injected intravenously with sufficient stable T_3 to saturate the nuclear receptor sites completely (25 μ g of T_3 /100 g of body wt). Animals were killed 90 min later, pituitary nuclei were prepared, and saturation studies were performed under various conditions of time and temperature. Euthyroid animals injected with an equivalent volume of vehicle served as controls.

Determination of nuclear T_3 content. The amount of T_3 present in pituitary nuclei was determined by radioimmunoassay (RIA) according to the methods of Larsen et al. (28). Individual rat anterior pituitary glands were homogenized by hand in 1 ml of homogenate buffer. Isolated pituitary nuclei were prepared as described above and extracted twice with 0.5 ml of absolute ethanol at 37°C for 30 min. The nuclear pellet was then stored at –20°C for subsequent DNA analysis. The two extracts for a given sample were pooled and stored at –20°C for subsequent T_3 RIA. Extraction efficiency was determined by injecting euthyroid rats intravenously with “tracer” amounts of ^{125}I - T_3 (4 ng/100 g body wt) 60 min before killing. Recovery of ^{125}I - T_3 in the nuclear extract was $84.3 \pm 1.8\%$ in the first ethanol extract and $96.7 \pm 2.5\%$ after two extractions. NSB of ^{125}I - T_3 was 3% as noted earlier, thus no correction for extraction efficiency or NSB was applied.

Determination of T_4 5'-deiodinase activity. T_4 5'-deiodinase activity was determined in the cytosol fraction of the crude pituitary homogenate by modification of the methods of Kaplan (21). T_4 was obtained from Henning, Co. (Berlin, Federal Republic of Germany) and contained <0.1% T_3 by the manufacturer's specifications. Aliquots (190 μ l) of cytosol enriched with DTT to a final concentration of 20 mM were preincubated at 37°C for 10 min in stoppered plastic vessels. T_4 was then added as a 10- μ l aliquot in 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.6, to yield a final concentration of 1.4–100 nM. Duplicate 40- μ l samples of reaction mixture were then immediately removed for zero time samples and each mixed with 80 μ l of ice-cold 95% ethanol. Incubation was then carried out for 10–30 min at 37°C after which duplicate samples were again obtained. After storage at 4°C for 24–36 h, ethanol extracts were centrifuged at 12,000 g and the supernate fraction stored at –20°C until assayed for T_3 by RIA. Recovery of T_3 by ethanol extraction of the reaction mixture was >96%. Protein was determined on aliquots of cytosol before enrichment with DTT by the method of Lowry et al. (29). Kinetic data were analyzed by Eadie-Hofstee plots (30).

RIA methods. T_3 concentrations in ethanol extracts and serum were determined by RIA by modification of the procedures of Larsen et al. (28, 31). A 5-d, nonequilibrium assay method with a combined polyethylene-glycol/second antibody separation step was employed. For cytosolic ethanol extracts from T_4 5'-deiodinase experiments, the assay buffer consisted of 0.2 M glycine, 0.13 M sodium acetate, pH 8.6, with 20 mg/dl bovine serum albumin. Extract aliquots (10 μ l) were assayed directly and equivalent volumes of ethanol/homogenate buffer were added to the standard curve. Specifically-bound ^{125}I - T_3 in the “zero” binding tubes ranged from 25 to 35%. NSB in the standard tubes was <2.0%. In sample tubes NSB was 2–4% and was determined for representative samples in all assays. Assay sensitivity, defined as the dose of T_3 resulting in ^{125}I - T_3 displacement from antibody greater

than three standard deviations from the zero binding, varied from 0.2 to 0.6 pg/tube. The amount of T_3 resulting in 50% displacement of tracer from zero binding was 3–6 pg/tube. Logit-log plots of the standard curve were linear from 0.2 to 30 pg/tube. Intra- and interassay coefficients of variation were 6% and 9%, respectively, at 3 pg of T_3 /tube.

Before assay, nuclear ethanol extracts were dried under N_2 at 37°C and then reconstituted in 100 μ l of 0.1 N NaOH, 565 μ l RIA assay buffer, and 35 μ l of human serum previously treated with an ion exchange resin (32). Recovery of T_3 from the dried sample was >99%. Duplicate 200- μ l aliquots of this mixture were then assayed for T_3 content with a third aliquot used to determine NSB. For these assays 10 μ l of resin-treated human serum was added to the standard curve and 2.74 g/dl sodium salicylate was included in the assay buffer. Assay sensitivity was 0.4–1.0 pg of T_3 /tube with 50% displacement of tracer occurring with 6–10 pg of T_3 /tube. Dilutions of nuclear extracts over an eightfold range were parallel to the standard curve. The T_3 content of nuclear extracts from control, fasting, and hypothyroid animals as determined by RIA with our standard separation step was compared with values obtained with a charcoal separation step optimized according to the methods of Odell (33). The coefficient of variation for five samples determined by the two techniques was <5%.

For determining serum T_3 and T_4 concentrations, 1 g/dl sodium salicylate was included in the assay buffer (31). Sample size was 10 μ l of serum in the T_3 RIA and 2 μ l in the T_4 RIA and an equivalent volume of thyroid hormone depleted rat serum (32) was added to the standard curves. Assay sensitivity was \sim 1 pg of T_3 /tube and 5 pg of T_4 /tube.

TSH was assayed using materials kindly supplied by the National Institute of Arthritis, Digestive and Kidney Diseases by Dr. A. Parlow. Results are expressed in terms of the rat TSH RP-1 standard.

All results are given as mean \pm standard error (SE). Data were analyzed using the *t* test for paired and unpaired values and two-variable linear regression analysis.

Results

Animals fasted for 72 h lost an average of 22% of their initial body weight compared with an 11% increase in weight of control animals. In addition, pituitary wet weight was 21% lower in fasted rats (7.3 ± 0.1 mg/pituitary control vs. 5.8 ± 0.1 mg/pituitary fasting, $P < 0.001$) and total pituitary cytosolic protein content was decreased by 13% ($P < 0.1$). The ratio of cytosolic protein to DNA content, however, was similar in control, fasting, and hypothyroid animals (mean value: 13.8 ± 0.8 mg of cytosolic protein/mg of DNA).

Serum T_3 , T_4 , TSH levels. Serum total T_3 , T_4 , and TSH levels for animals from the two experimental protocols are shown in Table I. In fasted animals (protocol I), serum T_3 and T_4 levels were decreased to 48 and 57% of control values, respectively. Fasting also resulted in a 48% reduction in serum TSH levels. In experiments involving hypothyroid and T_4 -injected animals (protocol II), serum T_3 and T_4 levels were lower in control rats than in the protocol I control animals (T_3 $P < 0.001$, T_4 $P < 0.025$) whereas TSH levels were higher ($P < 0.05$). In thyroidectomized animals serum T_3 and T_4 levels were decreased to 47 and 12% of control values whereas TSH levels were increased 10-fold. In rats receiving T_4 injections, both serum levels of T_3 and T_4 were elevated approximately threefold whereas TSH levels were decreased by 83%.

Technical aspects of saturation analysis. The kinetics of 125 I- T_3 binding to isolated pituitary nuclei from euthyroid rats was investigated at both 22 and 37°C. At 22°C (Fig. 1 A), maximal binding occurred between 10 and 20 h of incubation and then declined. Approximately 8 h was required for 50%

Table I. Serum T_3 , T_4 , and TSH Levels*

	T_3	T_4	TSH
	ng/dl	μ g/dl	ng/ml
Protocol I			
Control (15)	77 ± 5	6.0 ± 0.3	282 ± 40
Fasting (15)	$37 \pm 4^\ddagger$	$3.4 \pm 0.3^\ddagger$	$147 \pm 24^\S$
Protocol II			
Control (13)	49 ± 3	5.2 ± 0.2	449 ± 57
Hypothyroid (14)	$23 \pm 2^\ddagger$	$0.6 \pm 0.1^\ddagger$	$4490 \pm 318^\ddagger$
T_4 -injected (9)	$131 \pm 18^\ddagger$	$17.7 \pm 4.2^{ }$	$76 \pm 21^\ddagger$

* The data are presented as the mean \pm SE. (n) = number of rats per group.

‡ $P < 0.001$ compared with control.

§ $P < 0.01$ compared with control.

$^{||}$ $P < 0.02$ compared with control.

dissociation of bound 125 I- T_3 . After 3.5 h only 30% was dissociated. A comparably slow rate of dissociation (25% at 3.5 h) was noted in vitro at 22°C when pituitary nuclei were labeled in vivo with “tracer” amounts of 125 I- T_3 . With prolonged

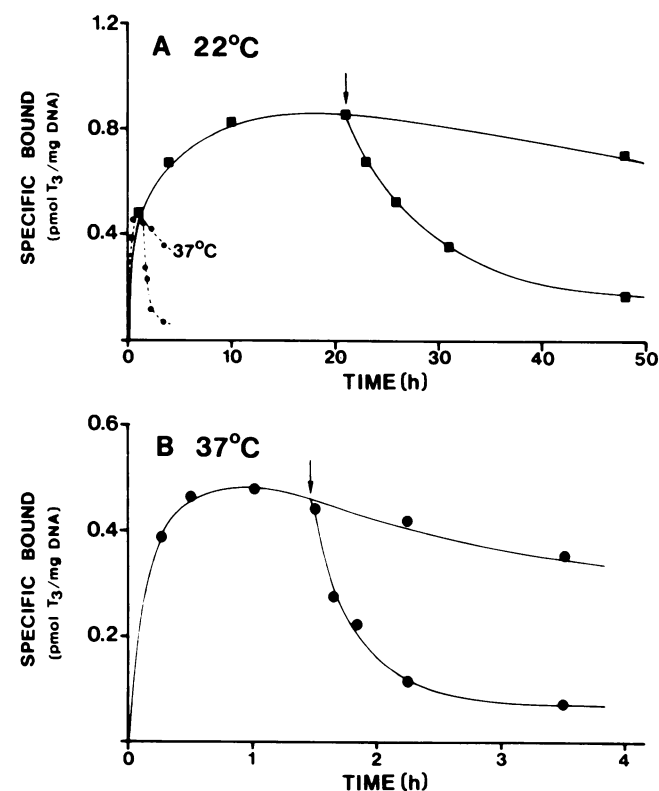


Figure 1. Time course of in vitro association/dissociation of 125 I- T_3 with isolated pituitary nuclei from euthyroid rats. Anterior pituitary glands from 22 male rats (200–225 g) were pooled and a purified suspension of nuclei was prepared as described. Aliquots of the suspension were then incubated for varying periods of time with 10^{-10} M 125 I- T_3 at either (A) 22°C or (B) 37°C. At the times indicated by the arrows, stable T_3 (final concentration 5×10^{-7} M) was added to some of the incubation tubes. NSB at both incubation temperatures was assessed by incubating aliquots of the nuclear suspension beginning at time zero with both 125 I- T_3 and 5×10^{-7} M stable T_3 . The data at 37°C depicted in (B) are reproduced in (A) for comparison.

in vitro incubation (30–50 h), 80% of either the in vivo- or in vitro-bound ^{125}I - T_3 dissociated. Binding kinetics at 37°C were considerably more rapid (Fig. 1 B); near-maximal binding was achieved after 30 min of incubation and only 20 min was required for 50% dissociation of bound hormone. Total binding at 37°C , however, was only 56% of that at 22°C reflecting a temperature-dependent decrease in receptor affinity as well as probable greater receptor instability.

The slow dissociation rate at 22°C indicated that saturation studies carried out at this temperature for relatively brief incubation periods (3.5 h) would primarily determine unoccupied receptor sites. To confirm this, saturation experiments were performed in isolated pituitary nuclei from euthyroid animals injected 90 min prior to killing with a large dose of stable T_3 (25 μg of T_3 /100 g of body wt). As shown in Fig. 2 A, Scatchard analysis after 3.5 h of incubation revealed a very low, apparent maximum binding capacity (MBC) in the T_3 -injected animals compared with vehicle-injected controls (MBC 0.2 vs. 1.4 pmol T_3 /mg DNA, respectively). More prolonged periods of incubation (27 h) resulted in higher apparent MBC values in nuclei from T_3 injected animals (MBC 1.2 pmol T_3 /mg DNA) demonstrating exchange of ^{125}I - T_3 with endogenously bound hormone.

The possibility was considered that the low apparent MBC at 3.5 h of incubation observed in T_3 -injected animals was secondary to a high nuclear T_3 content, contributing substantial quantities of hormone to the in vitro saturation analysis. This would result in higher total T_3 concentrations and decreased ^{125}I - T_3 binding. However, direct measurement of nuclear T_3 by RIA revealed levels in T_3 -injected animals of ~ 3 pmol T_3 /mg DNA, an amount that would increase the total mass of T_3 in the tubes containing the high concentrations of T_3 by $<10\%$ and thus not significantly affect the MBC. The substantial increase in the apparent MBC at 27 h in T_3 -injected animals provides a further argument against an artifactual lowering of the MBC by this mechanism. In addition, the latter result precludes the possibility that the low apparent MBC noted after 3.5 h of incubation was due to a rapid decrease in receptor number in vivo after the T_3 injection.

Scatchard analysis of saturation experiments performed at an incubation temperature of 37°C (Fig. 2 B) revealed a similar MBC in nuclei from animals injected either with vehicle or large doses of T_3 , confirming the rapid dissociation rate noted

in the kinetic experiments. However, despite complete dissociation of endogenous hormone at 37°C , the MBC of ~ 1 pmol T_3 /mg DNA was not greater than that determined at 22°C . This implied a significant and relatively rapid loss of receptor binding capacity at 37°C .

In contrast to the effects of prolonged incubation at 22°C with nuclei from T_3 -injected animals, incubation of nuclei from vehicle-injected or euthyroid control animals for periods longer than 3.5 h did not significantly increase the MBC. As shown in Fig. 3, the apparent MBC in euthyroid nuclei increased from 0.98 to 1.15 pmol T_3 /mg DNA when in vitro incubation was extended from 1 to 3.5 h at 22°C . This increase in MBC of 0.17 pmol T_3 /mg DNA correlates well with a predicted increase of 0.21 pmol T_3 /mg DNA given an endogenous nuclear T_3 content of ~ 1.4 pmol T_3 /mg DNA (discussed later) and the fact that $\sim 15\%$ of the endogenously bound hormone should dissociate between 1 and 3.5 h of incubation. With a stable binding system, extension of the incubation period to 27 h should result in a further increase in the apparent MBC to ~ 2.0 pmol T_3 /mg DNA. Such an increase, however, was not observed either in the experiment depicted in Fig. 3 or in three additional experiments where the effects of incubation time on MBC were investigated. Presumably greater degrees of T_3 binding were not observed with the more prolonged incubation periods in euthyroid nuclei due to a relative balance between the rates of endogenous hormone dissociation and receptor degradation.

The results of the preceding experiments demonstrated that in vitro saturation techniques under the conditions employed could not accurately quantify total T_3 receptor capacity in isolated pituitary nuclei. However, the MBC determined from saturation studies carried out for relatively short incubation periods (3.5 h) at 22°C (MBC_{22°) appeared to reflect reliably the unoccupied receptor capacity. Because $\sim 25\%$ of endogenously bound hormone dissociates under these conditions, the following relationship was used to estimate the number of unoccupied receptor sites: unoccupied sites = $\text{MBC}_{22^\circ} - (0.25) \text{ occupied sites}$. Thus, the number of unoccupied sites and, hence, total receptor capacity could be estimated using data from the in vitro saturation studies (MBC_{22°) and values obtained by RIA for nuclear T_3 content.

Saturation studies at 22°C . Scatchard analysis of saturation studies performed at 22°C for a 3.5-h incubation period with

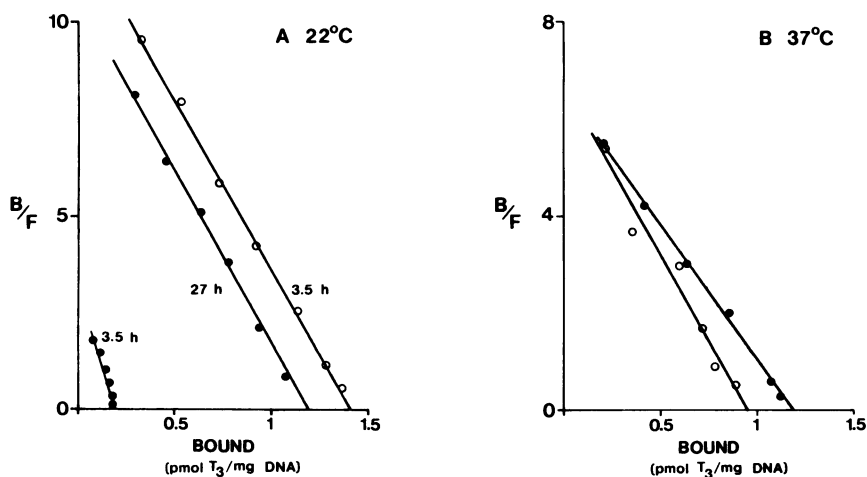


Figure 2. Scatchard analysis of in vitro binding data to isolated pituitary nuclei from euthyroid rats injected with a saturating dose of T_3 . (A) Male rats (200–225 g) were injected intravenously 90 min before sacrifice with either 25 μg of stable T_3 /100 g of body wt (15 animals) on an equal volume of vehicle (eight animals). Anterior pituitary glands from each group of rats were pooled, a suspension of isolated nuclei was prepared, and saturation analysis was performed at 22°C for either 3.5 or 27 h. (B) Incubation at 37°C for 2 h. Free concentrations are expressed in nanomolar. ●, T_3 -injected; ○, vehicle injected.

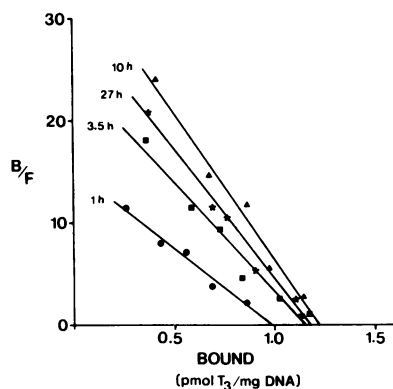


Figure 3. Effects of varying incubation time at 22°C on saturation analysis in isolated nuclei from euthyroid rats. Anterior pituitary glands from 32 male rats (200–225 g) were pooled, a suspension of isolated nuclei was prepared, and saturation analysis was performed at 22°C for 1, 3.5, 10, or 27 h. Values of K_d for each preparation are: 6.5×10^{-11} M (1 h), 4.7×10^{-11} M (3.5 h), 3.4×10^{-11} M (10 h), and 3.9×10^{-11} M (27 h). Free concentrations are expressed in nanomolar.

pituitary nuclei from all experimental groups revealed a single class of saturable, high-affinity binding sites. Representative experiments are shown in Fig. 4. As summarized in Table II, values of MBC_{22° obtained in control animals from protocol I were somewhat lower than control values noted in protocol II ($P < 0.01$). Fasting resulted in a 29% decrease in MBC_{22° , a decrease similar to that noted in two groups of T_4 -injected animals. In hypothyroid animals, MBC_{22° was increased by 42%. Values for the equilibrium dissociation constant (K_d) (Table II) were similar for all groups with a mean value of $7.2 \pm 0.75 \times 10^{-11}$ M ($n = 18$).

Nuclear T_3 content and estimation of total nuclear receptor capacity. Levels of receptor-bound endogenous T_3 are shown in Table II. The nuclear T_3 content of control animals from protocol I experiments was significantly higher than that noted in the protocol II control group ($P < 0.001$); a finding consistent with the higher serum T_3 and T_4 levels and lower TSH levels seen in the former group. In fasted animals, nuclear T_3 levels were significantly decreased; however, the decrease was relatively modest ($\sim 15\%$) given the much larger decreases noted in serum T_3 (52%) and serum T_4 (43%) levels. Thyroidectomized animals demonstrated a 61% decrease in nuclear T_3 content.

Because the MBC_{22° and nuclear T_3 content data were obtained in different groups of experimental animals, the mean

values of these parameters were utilized to calculate the unoccupied and total receptor binding capacities. As shown in Table II, these estimated values decreased 32 and 23%, respectively, during fasting. Although the nature of the experimental data prevents a statistical analysis of these calculated figures, the fact that they are derived from two experimental data sets, each of which showed statistically significant decreases with fasting, strongly suggests that these calculated values are also of significance. Estimates of receptor occupancy revealed that 62% of binding sites were occupied in fasted animals compared with a 56% occupancy rate in protocol I controls. The significance of this increase in T_3 receptor occupancy accompanying fasting can again only be inferred from the fact that the 29% decrease in MBC_{22° in fasted rats was approximately twice the decrease noted in nuclear T_3 content. Thus the decrease in unoccupied and total receptor sites was relatively greater than the decrease in occupied sites suggesting an actual increase in receptor occupancy.

In hypothyroid animals, the number of unoccupied nuclear T_3 binding sites was estimated to increase by $\sim 55\%$ as compared with protocol II controls. Total binding capacity was also calculated to be slightly higher in hypothyroid animals (2.7 vs. 2.3 pmol T_3 /mg DNA, respectively). The fact, however, that nuclear T_3 levels decreased and MBC_{22° increased by comparable amounts in the thyroidectomized group (0.5 pmol T_3 /mg DNA decrease in nuclear T_3 vs. 0.7 pmol T_3 /mg DNA increase in MBC_{22°) suggests that total binding capacity is unaltered in hypothyroidism. As expected from the marked decrease in nuclear T_3 content, receptor occupancy in hypothyroid animals was considerably lower than in control animals. Although nuclear T_3 content was not determined in T_4 -injected animals, one can estimate that given the marked decrease in MBC_{22° and assuming no change in total receptor number, nuclear occupancy in this group of animals would be $\sim 70\%$.

Confirmation of these estimates of total receptor capacity was sought by performing saturation analysis under such in vitro conditions that would allow complete dissociation of endogenous hormone (1-h incubation at 37°C). In two experiments, fasting resulted in a 25% and 42% decrease in MBC . These values provide additional evidence that the decrease in total receptor capacity during fasting is relatively greater than the decrease in nuclear T_3 content and thus support the previous estimates of an actual increase in receptor occupancy. Similar experiments utilizing thyroidectomized animals demonstrated a 36% increase in MBC in one experiment and a 7% decrease in another, thus confirming that total receptor

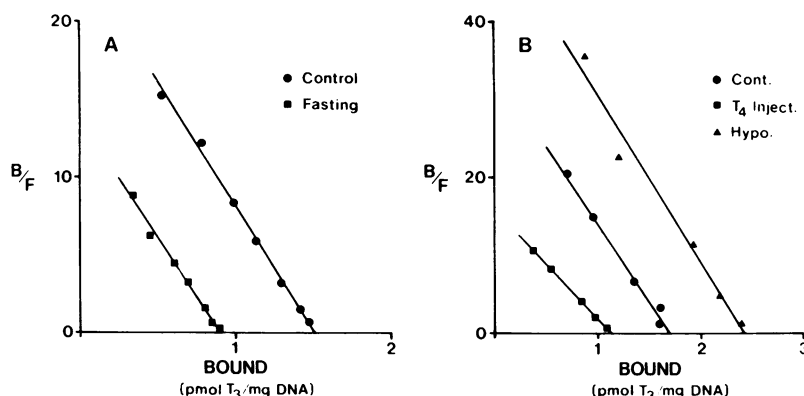


Figure 4. Scatchard analysis of in vitro binding data to isolated pituitary nuclei from (A) control and fasting animals (protocol I) and (B) control, T_4 injected, and thyroidectomized animals (protocol II). Anterior pituitaries from 4 to 11 animals in each experimental group were pooled, a suspension of isolated nuclei was prepared, and saturation analysis was performed at 22°C for 3.5 h of incubation. Free concentrations are expressed in nanomolar.

Table II. Results of Saturation Analysis at 22°C, Nuclear T₃ Content, and Estimation of Nuclear Receptor Capacity*

	Saturation analysis		Nuclear T ₃ content	Unoccupied receptor capacity	Total receptor capacity	Receptor occupancy
	K _d	MBC ₂₂ *				
	× 10 ⁻¹¹ M	pmol T ₃ /mg DNA	pmole T ₃ /mg DNA	pmol T ₃ /mg DNA	pmole T ₃ /mg DNA	%
Protocol I						
Control	7.1±1.1 (7)	1.48±0.04 (7)	1.43±0.06 (8)	1.11	2.54	56
Fasting	8.0±2.0 (3)	1.05±0.08 (3)‡	1.21±0.07 (7)§	0.75	1.96	62
Protocol II						
Control	6.5±0.8 (3)	1.73±0.05 (3)	0.79±0.04 (13)	1.53	2.32	34
Hypothyroid	4.4±0.5 (3)	2.45±0.17 (3)	0.33±0.07 (6)‡	2.37	2.70	12
T ₄ -injected	7.1, 15.0 (2)	1.13, 1.16 (2)	—	—	—	—

* Data are presented as mean±SE. (n) = number of experiments for K_d and MBC₂₂ and number of animals for nuclear T₃ content. Unoccupied receptor capacity calculated as MBC₂₂ - (0.25) nuclear T₃ content. Total receptor capacity calculated as nuclear T₃ content + unoccupied receptor capacity. Receptor occupancy calculated as (nuclear T₃ content ÷ total receptor capacity) × 100. Mean values of MBC₂₂ and nuclear T₃ content were used in these calculations. ‡ P < 0.005 compared with control. § P < 0.05 compared with control. Not included in the nuclear T₃ content data for fasting animals is the value in one animal, which was greater than 3 SD above the mean for the rest of the fasting group and greater than any value determined in the control group. Inclusion of this animal alters the mean fasting value very little (1.28±1.0 pmol T₃/mg DNA); however, statistical significance is lost (P = 0.2). ^{||} P < 0.02 compared with control.

number is either unchanged or somewhat increased in hypothyroid animals. The average value of the K_d determined by Scatchard analysis in these experiments was twice the value noted when saturation analysis was performed at 22°C (14.2±1.53 × 10⁻¹¹ M [n = 8] vs. 7.2±0.75 × 10⁻¹¹ M [n = 18] respectively, P < 0.001).

T₄ 5'-deiodinase activity. The time course of T₄ 5'-deiodination in the cytosol fraction of the homogenate from euthyroid pituitaries is shown in Fig. 5. The reaction rate was linear for the first 5 min of incubation and then slowly decreased. At 10, 14, and 30 min, the reaction rates were 90, 81, and 61%, respectively, of the initial velocity noted at 5 min. Incubation times utilized in subsequent experiments were 14–15 min in control and fasted animals, 10 min in hypothyroid animals, and 30 min in T₄-injected animals. These times were designed to minimize substrate utilization and yet allow for sufficient T₃ accumulation for accurate measurement by RIA. Correction factors for these varying incubation times were not applied. In other experiments, aliquots of cytosol kept at 0–4°C maintained >90% of activity for 1 h. The coefficient of variation of

replicate determinations at low substrate concentrations was <15% and at high substrate concentrations <10%. The rate of T₃ formation was found to increase linearly with homogenate protein concentration up to 6 mg/ml. Protein content of the crude cytosolic preparation was typically 2–3 mg/ml. Incubation of homogenates from both control and fasted animals with known concentrations of stable T₃ and 20 mM DTT for 30 min at 37°C failed to disclose any evidence of T₃ degradation. No evidence of T₃ formation was noted in cytosol fractions that were either heated to 80°C for 45 min or were not enriched with DTT.

Using substrate concentrations of 1.4–100 nM T₄ and 20 mM DTT, 5'-deiodination of T₄ displayed simple saturation kinetics. Eadie-Hofstee plots (Fig. 6) revealed apparent Michaelis constant (K_m) values of ~4 nM in both control and fasting animals, reflecting the low K_m (type II) 5'-deiodinase component responsible for the majority of T₄ to T₃ conversion

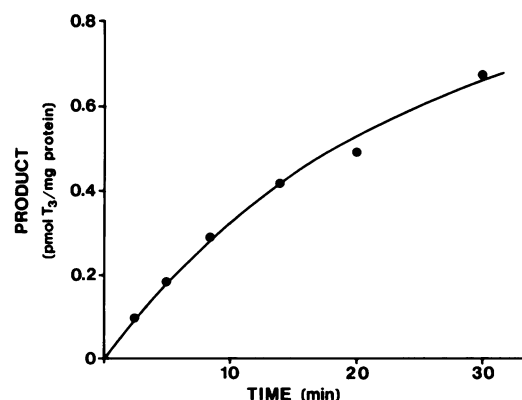


Figure 5. Time course of T₃ formation from T₄ 5'-deiodination in the cytosol fraction of euthyroid pituitary homogenates. Substrate concentrations were 9 nM T₄ and 20 mM DTT. Points represent the mean of duplicate determinations, which varied by <10%.

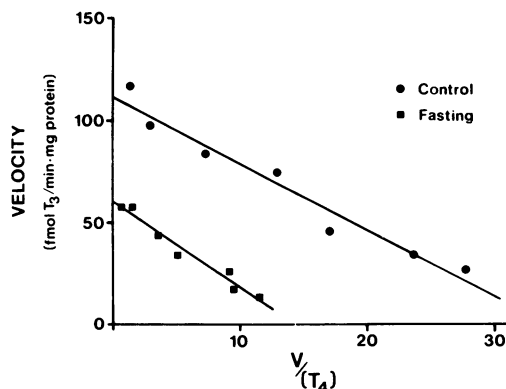


Figure 6. Kinetic analysis of pituitary T₄ 5'-deiodinase activity in control and fasting rats by use of the Eadie-Hofstee plot. Aliquots of the pooled cytosol fraction from pituitary homogenates were incubated with 1.4–100 nM T₄ and 20 mM DTT for 14–15 min. T₃ formation was then quantified in ethanol extracts of the reaction mixture by RIA. T₄ concentrations are expressed in nanomolar.

in the pituitary (34). As shown in Table III, fasting was associated with a 37% decrease ($P < 0.025$) in maximal enzyme activity (V_{\max}). In hypothyroid animals, V_{\max} increased approximately ninefold, whereas in T_4 -injected animals, 5'-deiodinase activity was reduced to $\sim 10\%$ of the value seen in control animals.

Discussion

The principal goal of the present study was to compare the intracellular factors affecting thyroid hormone action in the pituitary during the fasting and hypothyroid states. Because the interaction of T_3 with specific, high-affinity nuclear-binding sites appears to represent an important event in the control of TSH secretion (35–37), we sought to quantify pituitary T_3 -generating activity and the number and degree of occupancy of nuclear thyroid hormone receptors. The application of in vitro receptor-binding techniques to isolated pituitary nuclei was complicated significantly by receptor occupancy with endogenous hormone, slow dissociation of the hormone-receptor complex at 22°C, and receptor instability at both 22 and 37°C. This combination of factors can obviously result in serious misinterpretation of in vitro binding data. The attainment of a plateau in the association curve, as demonstrated between 10 and 21 h at 22°C and 30 and 90 min at 37°C (Fig. 1), is clearly insufficient evidence that both stability and equilibrium conditions exist so that total receptor capacity can be determined accurately by Scatchard analysis.

The justification for applying Scatchard analysis in these experiments where nonsteady-state conditions exist is based on the rapid attainment of equilibrium with the unoccupied receptor fraction. As discussed by Aranyi (38) and Yeakley et al. (39), lack of equilibrium with unoccupied binding sites results primarily in underestimation of receptor affinity with only minimal error in the determination of unoccupied binding capacity. The high receptor affinity noted at 22°C ($K_d \sim 7 \times 10^{-11}$ M), as well as the lack of significant change in the K_d when incubation is extended from 3.5 to 27 h (Fig. 3), strongly suggests that equilibrium with unoccupied receptor sites occurs rapidly.

A somewhat surprising finding in this study was the significant differences noted between protocol I and protocol

II control groups in essentially all experimental parameters. These differences were not due to interassay variation in that values for the various serum parameters for all experimental groups were determined in the same assays. In addition, experimental values for control groups within a single protocol were remarkably consistent as shown in Tables I–III. Although experiments involving animals in protocols I and II were not performed on the same day, experiments were frequently carried out concurrently, and thus there was no significant difference in the time frame of data acquisition. Of possible importance, however, is the fact that protocol I control animals were somewhat younger at the time of sacrifice than protocol II controls (8 wk vs. 11 wk, respectively) and were housed under potentially more stressful conditions (as discussed earlier). These factors may have contributed to the differences observed in the experimental data between the two groups.

Experimental findings and physiologic implications. Although thyroid hormones are of unquestionable importance in controlling various pituitary functions, the cellular mechanisms mediating these effects remain poorly understood. Recent evidence suggests that the regulatory effects of T_3 on TSH secretion may be related to the degree of occupancy of pituitary nuclear T_3 receptors (35–37). Our finding that hypothyroidism is accompanied by a 61% decrease in nuclear T_3 content and a marked decrease in receptor occupancy is consistent with this hypothesis and confirms work by previous investigators (28, 40). In fasted rats, however, a strikingly different situation is seen despite significant decreases in both serum T_3 and T_4 concentrations. Thus, although nuclear T_3 levels in starved animals are also decreased, the decrease is relatively modest (15%) and when combined with the 23% reduction in total binding capacity, receptor occupancy appears to be unchanged or actually increased relative to control values. If indeed receptor occupancy (irrespective of total receptor number) is the key parameter mediating thyroid hormone regulation of TSH secretion, then the present results offer a plausible explanation for the failure of TSH levels to rise during short-term fasting in the rat. Support for this concept can be obtained from the present studies. For the five experimental groups examined, an excellent correlation was noted between the estimated receptor occupancy and the mean serum TSH levels as shown in Fig. 7 ($r = -0.97$, $P < 0.01$). Although other factors not specifically addressed in these studies, such as alterations in hypothalamic secretion or postreceptor events mediated directly by fasting, may also be important in the response of the pituitary to starvation, the maintenance of relatively normal nuclear T_3 levels and normal to increased receptor occupancy are likely to be important contributing factors to the observed suppression in circulating TSH levels.

Our data regarding thyroid hormone economy in the pituitary during fasting are in sharp contrast to the findings reported by others in the liver (8). Thus, short-term starvation in the rat is accompanied by profound decreases in hepatic nuclear T_3 levels which, despite a decrease in total receptor number, result in a significantly decreased receptor occupancy rate (8, 41, 42). Accompanying these observed changes are metabolic alterations in the liver and other peripheral tissues that appear to be due in part to decreased thyroid hormone action (1, 6, 9–11, 17, 43). In this regard, the decrease in hepatic T_4 5'-deiodinase activity (high K_m , type I) noted in fasting (44) is similar to that occurring in hypothyroidism (22) and can largely be reversed by the administration of thyroid

Table III. Pituitary T_4 5'-Deiodinase Activity*

	K_m	V_{\max}
	nM	fmol T_3 /min · mg prot
Protocol I		
Control (4)	4.3±0.5	95±11
Fasting (5)	4.0±0.4	60±6§
Protocol II		
Control (3)	5.2±1.2	57±9
Hypothyroid (3)	7.0±1.8	491±59
T_4 -injected (2)‡	—	7, 3

* Data are presented as mean±SE. (n) = number of experiments.

‡ Velocity values for T_4 -injected animals were determined at a single T_4 concentration of 100 nM. Enzyme activity was too low to permit determination of K_m .

§ $P < 0.025$ vs. control.

^{||} $P < 0.005$ vs. control.

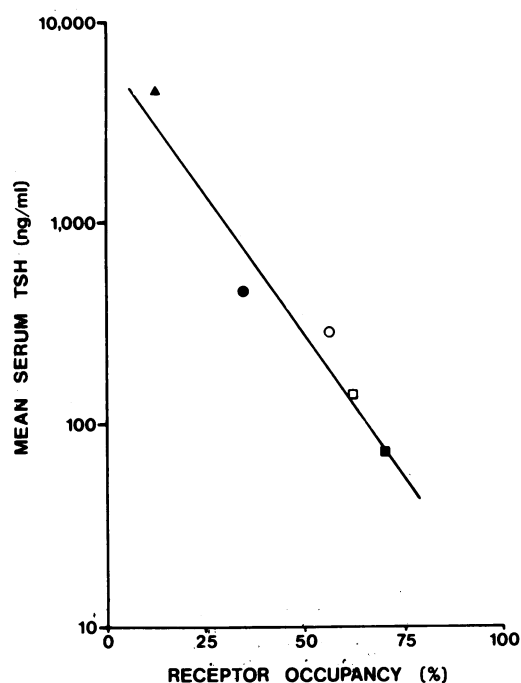


Figure 7. Mean serum TSH concentrations plotted as a logarithmic function of the estimated T_3 receptor occupancy for the five experimental groups. Receptor occupancy in T_4 -injected animals was calculated by assuming a total receptor number equal to that in protocol II control animals. Control I, \circ ; fasting, \square ; control II, \bullet ; hypothyroid, \blacktriangle ; T_4 -injected, \blacksquare ; $r = -0.97$, $P < 0.01$.

hormone during the fasting period (45, 46). In contrast, the decreased pituitary T_4 5'-deiodinase activity (low K_m , type II) noted in the present study is qualitatively similar to that seen in the T_4 -injected animals (21) and thus suggests an enhanced action of thyroid hormone on the hypophysis during fasting, a formulation that is consistent with the observed decreases in serum TSH levels and the increases noted in nuclear receptor occupancy.

The factors responsible for the maintenance of near normal pituitary nuclear T_3 levels in the fasted rat are uncertain but of potentially great physiologic importance. Although in vivo rates of T_4 to T_3 conversion were not determined in the present study, one can infer from the observed decreases in serum T_4 levels and in vitro 5'-deiodinase activity that "local" T_3 generation by the pituitary as a whole is diminished during fasting. Mechanisms that could serve to "protect" nuclear T_3 levels in the fasting state include an increase in the rate of cellular or nuclear T_3 entry, a decrease in the fractional rate of cellular T_3 degradation, or a decrease in the turnover rate of occupied T_3 receptors. Additional studies will be required to address these possibilities.

Comparison with prior studies. A comparison of the present data with previous studies reveals apparent discrepancies in the values for T_3 receptor number and occupancy. Others, by using in vivo saturation techniques, have estimated that the binding capacities of euthyroid and hypothyroid rat pituitary nuclei are 1.2 and 1.5 pmol T_3 /mg DNA, respectively (18, 36, 47), values that are 50% lower than those determined in the present study. Similar discrepancies have been noted between data obtained in vivo and in vitro in cerebral cortex (48). Although the reasons for these differences are uncertain, several

possibilities exist. An important assumption inherent to the in vivo technique is that equilibration has been achieved between serum and nuclear T_3 (49). Recent evidence, however, suggests that the entry of T_3 to both the cytoplasm and the nucleoplasm may be a receptor-mediated, saturable process (50–52). Thus significant intracellular free T_3 gradients may exist (52) and equilibration between serum, cytoplasm, and nucleus may be delayed at the higher serum T_3 levels employed during in vivo saturation studies. Furthermore, given that the time for 50% dissociation of the T_3 -receptor complex in vivo may be as long as 1 h (53), it is unlikely that the rather brief period of time during the in vivo analysis when serum and nuclear ^{125}I - T_3 levels are in steady state is sufficient for a true equilibrium to occur with previously occupied receptors. Given these uncertainties, it is possible that the two-compartment model (54), which forms the basis for the in vivo saturation techniques, represents an oversimplification and results in the measurement of only a fraction of the total complement of nuclear receptors. Alternatively, it is possible that the procedures utilized herein in preparing the nuclei for the in vitro studies expose additional nuclear T_3 -binding sites not available for in vivo binding. This appears unlikely, however, because in vivo injection of a large dose of T_3 reduced MBC_{22} to a very low value (Fig. 2 A), indicating complete receptor saturation. Regardless of the reasons for these differences, however, our finding of a significant number of unoccupied binding sites in hyperthyroid animals (Table II) and a large discrepancy in apparent MBC (after 3.5 h of incubation) in nuclei from vehicle-injected and in vivo T_3 -saturated animals (Fig. 2 A) strongly suggests that endogenous pituitary receptor occupancy in the euthyroid rat is less than the 78% value reported by Silva et al. (55), who used in vivo techniques.

The application of in vitro saturation techniques to pituitary tissue has been limited (56). Recently, Coulombe et al. (57) and von Overbeck and Lemarchand-Beraud (58) have reported a significant decrease in the number of solubilized pituitary nuclear T_3 receptors in adult hypothyroid rats; an observation that is in marked contrast to the present data and that noted previously by Silva and Larsen (36), who used in vivo techniques and by Samuels et al. (59) in T_3 -depleted growth hormone (GH_1) cells. Neither Coulombe et al. (57) or von Overbeck and Lemarchand-Beraud (58), however, adequately address the issues of receptor stability, association/dissociation rates, establishment of equilibrium with endogenously occupied sites, or efficiency of the receptor extraction technique. Indeed, it appears likely, from data presented by the latter investigators, that the solubilization methods employed preferentially extract occupied T_3 -binding sites. Furthermore, Seelig et al. (60) have recently demonstrated that binding of T_3 to solubilized hepatic nuclear receptors at 0–4°C does not follow a reversible bimolecular process, thereby making Scatchard analysis of such data invalid. Methodologic problems inherent to the study of salt-solubilized receptors are thus a possible explanation for the discrepancies noted above.

Pituitary 5'-deiodinase activity in fasted animals has been reported to be unchanged (19, 20) or decreased (21, 22). Previous investigators, however, by using a single relatively high concentration of T_4 ($\sim 1 \mu M$) during in vitro assay, have not been able to distinguish between high K_m (type I) and low K_m (type II) 5'-deiodinase activity. The kinetic data presented herein clearly demonstrate that fasting is accompanied by a significant decrease in V_{max} for the low K_m 5'-deiodinase

pathway with no change in K_m . This finding is in sharp contrast to the marked elevations in low K_m 5'-deiodinase activity noted in hypothyroid animals and suggests that factors in addition to peripheral thyroid hormone levels are involved in regulating this physiologically important enzymatic process.

The relevance of our findings in whole pituitary homogenates to the subset of TSH-producing cells is at present uncertain. Nevertheless, our data demonstrate that, in the fasting state, alterations occur in thyroid hormone economy in the pituitary, which serve to maintain relatively normal amounts of nuclear receptor bound T_3 and may result in an actual increase in receptor occupancy. We hypothesize that this mechanism may be responsible, at least in part, for the observed suppression of TSH secretion that accompanies short-term starvation in the rat.

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