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# Uptake of 3,3′,5,5′-Tetraiodothyroacetic Acid and 3,3′,5′-Triiodothyronine in Cultured Rat Anterior Pituitary Cells and Their Effects on Thyrotropin Secretion*

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**ABSTRACT**

We compared the uptake, metabolism, and biological effects of tetraiodothyroacetic acid (Tetrac) and $rT_3$ in anterior pituitary cells with those of $T_4$ and $T_3$. Cells were isolated from adult male Wistar rats and cultured for 3 days in medium with 10% fetal calf serum. Uptake was measured at 37 C in medium with 0.1% BSA for [$^{125}$I]Tetrac (200,000 cpm; 240 pM) and [$^{125}$I]$T_4$ (100,000 cpm; 175 pM) or with 0.5% BSA for [$^{125}$I]$rT_3$ (100,000 cpm; 250 pM) and [$^{125}$I]$T_3$ (50,000 cpm; 50 pM). The free fraction of Tetrac was 1% that of $T_4$ (in medium with 0.1 and with 0.5% BSA), and the free fraction of $rT_3$ was half that of $T_3$. Uptake of the four tracers increased sharply up to 1 h of incubation and then leveled off. Expressed as femtomoles per pM free hormone, uptake at equilibrium was 1.16 ± 0.16 (n = 6) for Tetrac, 0.15 ± 0.01 (n = 6) for $T_4$, 0.023 ± 0.003 (n = 6) for $rT_3$, and 0.21 ± 0.02 (n = 6) for $T_3$. Cell-associated radioactivity after incubation for 24 h with [$^{125}$I]Tetrac was represented for 15% by [$^{125}$I]Triac; after incubation with [$^{125}$I]$T_4$ for 15–20% by [$^{125}$I]$T_3$, after incubation with [$^{125}$I]$rT_3$ for 6% by [$^{125}$I]3,3′-$T_2$, while [$^{125}$I]$T_3$ was still for 98% [$^{125}$I]$T_3$. Exposure of cells for 2 h to 100 nM TRH stimulated TSH release by 90–135%. Tetrac was effective in reducing this response at a free concentration of 0.05 pM, but $rT_3$ was effective only at a free concentration of 16 nM. A free Tetrac concentration of 5 pM was equally effective as 50 pM free $T_4$ in reducing the TSH response to TRH. In human serum, Tetrac was exclusively bound to $T_4$-binding prealbumin. The free Tetrac fraction was 0.001% in control subjects and rose 2- to 12-fold in patients with nonthyroidal illness. As uptake of [$^{125}$I]Tetrac in the pituitary was higher than that of $T_4$ and $T_3$, and it was more potent than $T_4$ in reducing TSH release, Tetrac may be of potential significance for the regulation of TSH secretion *in vivo*. (*Endocrinology* **136:** 4454–4461, 1995)

NONTHYROIDAL illness (NTI) and fasting are characterized by a low serum $T_3$ concentration and an elevated serum concentration of $rT_3$, whereas the serum $T_4$ concentration is normal or eventually decreased (1–4). In the face of the low serum $T_3$ and $T_4$ concentrations, the serum TSH level is inappropriately low (1, 2, 4, 5).

Recently, it was proposed that changes in $T_4$ metabolism during fasting and NTI, *i.e.* a shift from deiodination to increased conjugation or alanine side-chain alteration, may result in metabolites with a TSH-suppressing effect. The first to be evaluated was $T_3$ sulfate (6), but this metabolite appeared to be biologically inactive at the pituitary level (7, 8), perhaps because it does not enter the tissue (8). The second to be tested was the acetic acid derivative of $T_3$, triiodothyroacetic acid (Triac) (9, 10), which is rapidly taken up by the pituitary (11) and has a profound TSH-suppressing effect both *in vitro* (11, 12) and *in vivo* (13, 14). Like Triac, the acetic acid derivative of $T_4$, tetraiodothyroacetic acid (Tetrac), was suggested to contribute to the suppression of TSH secretion during fasting and NTI (10). Administration of Tetrac reduced the TSH response to TRH in euthyroid subjects (13). Furthermore, in fasting subjects, the serum Tetrac concentration was reported to increase 2-fold (15).

Although $rT_3$ is not ascribed any biological activity, recent studies indicate that one of its metabolites 3,3′-diiodothyronine (3,3′-$T_2$) stimulates the mitochondrial respiratory rate (16). A similar action has been reported for 3,5-$T_2$ (a product of $T_3$ deiodination) (16, 17), which, in addition, has been shown to suppress TSH secretion in rats (18). The pituitary contains the enzyme that deiodinates $rT_3$ (19), but before this can occur, $rT_3$ has to enter the pituitary cells.

The present study was undertaken to investigate 1) the uptake and metabolism of $^{125}$I-labeled $rT_3$ and Tetrac in cultured anterior pituitary cells compared to that of $^{125}$I-labeled $T_3$ and $T_4$; 2) the effects of unlabeled Tetrac and $rT_3$ on the uptake of [$^{125}$I]$T_3$; and 3) the effects of unlabeled Tetrac, $rT_3$, 3,3′-$T_2$, and 3,5-$T_2$ on TRH-induced TSH release. Finally, we evaluated the distribution of Triac and Tetrac among serum proteins as well as the free fraction of Triac and Tetrac in serum of control subjects and NTI patients.

## Materials and Methods

### *Materials*

All solutions used for cell isolation and cell culture were obtained from GIBCO Europe (Breda, The Netherlands), with the exception of human serum albumin (Central Laboratory of the Red Cross Blood

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Transfusion Service, Amsterdam, The Netherlands), dispase (grade II; Boehringer Mannheim, Mannheim, Germany), and fungizone (Bristol-Myers Squibb, Woerden, The Netherlands). Culture dishes (48 wells) were obtained from Costar (Cambridge, MA). TRH was obtained from Hoechst (Frankfurt am Main, Germany). All iodothyronines, 3,5-diiodothyroacetic acid (3,5-Diac), Triac, and Tetrac, were obtained from Henning Berlin (Berlin, Germany). Piperazine-*N,N'*-bis-[2-ethane sulfonic acid] (PIPES), HEPES, *N,N*-bis-[2-hydroxyethyl]2-aminoethane sulfonic acid (Bes), 6-propyl-2-thiouracil (PTU), and BSA (fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO). [3',5'-$^{125}$I]$T_4$ (1500 $\mu$Ci/$\mu$g), [3'-$^{125}$I]$T_3$ (3070 $\mu$Ci/$\mu$g), [3',5'-$^{125}$I]$rT_3$ (1200 $\mu$Ci/$\mu$g), and carrier-free $Na^{125}I$ were purchased from Amersham International (Aylesbury, UK). HPLC pure [3'-$^{125}$I]Triac) (2730 $\mu$Ci/$\mu$g) and [3',5'-$^{125}$I]Tetrac (2200 $\mu$Ci/$\mu$g) were prepared from $Na^{125}I$ and 3,5-Diac or Triac, respectively, using the chloramine-T method followed by purification on Sephadex LH-20 (20). Sephadex LH-20 and G-25 were obtained from Pharmacia (Uppsala, Sweden). All other reagents were of the highest purity available.

### *Animals*

All experiments were performed using male Wistar rats, weighing 220–250 g. The animals had free access to food and water and were kept in a controlled environment (21 C) with constant day length (12 h).

### *Cell culture*

Animals (12–18 for each experiment) were killed between 0900–0930 h by decapitation. The pituitary glands were removed within 5 min, the neurointermediate lobe was discarded, and the anterior lobes were collected in calcium- and magnesium-free Hanks' Balanced Salt Solution supplemented with 10 g/liter human serum albumin, penicillin ($10^5$ U/liter), fungizone (0.5 mg/liter), and sodium bicarbonate (0.4 g/liter). Anterior pituitary cells were dissociated with dispase (final concentration, $2.4 \times 10^3$ U/liter) for 2 h at 37 C in a shaking incubator. From each pituitary, around $1.5 \times 10^6$ cells were obtained, and the viability of the cells, as determined by trypan blue exclusion, was greater than 90%.

The cells were cultured at 37 C in a water-jacketed incubator with 5% $CO_2$ at a density of 500,000 cells/well in 48-well culture dishes. The cells had attached to the wells after 2 days of culture. On day 3, the cells were used for experiments. The culture medium consisted of Minimal Essential Medium with Earle's salts supplemented with nonessential amino acids, sodium pyruvate (1 mmol/liter), 10% fetal calf serum, penicillin ($10^5$ U/liter), fungizone (0.5 mg/liter), L-glutamine (2 mmol/liter), and sodium bicarbonate (2.2 g/liter), pH 7.4 (21–23).

### *Cellular uptake of $^{125}$I-labeled $rT_3$, $T_3$, Tetrac, and $T_4$*

After removal of the culture medium, cells were preincubated with 0.5 ml incubation medium for 30 min at 37 C. The incubation medium was identical to the culture medium, except that fetal calf serum was replaced by 0.5%, 0.1%, or 0.01% BSA. After preincubation the medium was removed, and incubation (15 min–4 h) was started with 0.25 ml medium containing [$^{125}$I]$rT_3$ (100,000 cpm; 250 pM), [$^{125}$I]$T_3$ (50,000 cpm; 50 pM), [$^{125}$I]$T_4$ (100,000 cpm; 175 pM), or [$^{125}$I]Tetrac (200,000 cpm; 240 pM). Incubations lasting for more than 1 h were performed at 37 C in humidified air with 5% $CO_2$. Incubations of shorter duration took place in a 37 C incubation chamber on a rotating device without $CO_2$. Therefore, the $NaHCO_3$ in the culture medium was replaced by an equimolar amount of HEPES (8.9 mM), PIPES (10.6 mM), and Bes (11.2 mM). After incubation, the medium was removed, and cells were washed with 1-ml volumes of ice-cold saline to remove tracer not bound to the cells. Cells were dissolved in 1 ml 0.1 N NaOH and counted for $^{125}$I activity in a 16-channel $\gamma$-counter (NE 1600, Nuclear Enterprises, Edinburgh, Scotland). The amount of $^{125}$I-labeled compound taken up was expressed as a percentage of the radioactivity added (percent dose). The same procedure was applied to the incubations without cells. All results are corrected for the amount of radioactivity retained in the wells without cells.

For HPLC analysis of metabolites, incubations were performed for 24 h with $2 \times 10^6$ cpm $T_3$ (2 nM), $T_4$ (3.5 nM), $rT_3$ (5 nM), or Tetrac (2.4 nM). Cells were further processed as described above.

### *LH-20 chromatography*

Aliquots of the incubation medium and cell extracts were chromatographed on Sephadex LH-20 (24). Iodide was eluted from the column with 4× 1 ml 0.1 N HCl. Subsequently, conjugates were eluted with 8× 1 ml $H_2O$, and finally, iodothyronines were removed from the column with 3× 1 ml 1% $NH_4OH$ in ethanol.

### *HPLC*

The solvent (1% $NH_4OH$ in ethanol) of samples prepurified on Sephadex LH-20 was evaporated under a stream of $N_2$ at 50 C. The residue was redissolved in the HPLC mobile phase and injected onto Chromspher $C_{18}$ columns (10 × 0.3 cm; Chrompack International, Middelburg, The Netherlands) fitted in a Waters HPLC system (Waters Associates, Milford, MA). Isocratic elution was performed with a 53:47 mixture of methanol in 0.02 M ammonium acetate (pH 4) at a flow rate of 0.9 ml/min for studies of [$^{125}$I]$T_4$ metabolism and with a 60:40 mixture of the same solutions at the same flow rate for studies of [$^{125}$I]Tetrac metabolism. The latter was performed to speed up elution of the acetic acid derivatives. Fractions of 0.3 min were collected and counted for radioactivity. The retention times of the four unlabeled reference compounds were determined with the two elution mixtures by monitoring the absorbance of the eluate at 254 nM (20). The procedure was applied to cell samples as well as to the blank samples (without cells) to allow correction for degradation of [$^{125}$I]$T_4$ or [$^{125}$I]Tetrac during evaporation and HPLC analysis.

### *TSH release*

After removal of culture medium, cells were washed once with incubation medium. The incubation medium was identical to the culture medium, except that the fetal calf serum was replaced by 0.5% BSA. Pituitary cells were preincubated (0.5 ml) for 2 h at 37 C in a water-jacketed incubator with 5% $CO_2$ in the absence or presence of variable concentrations (0.01 nM to 1 $\mu$M) of $T_3$, $T_4$, $rT_3$, Triac, Tetrac, 3,5-$T_2$, or 3,3'-$T_2$. The preincubation medium was discarded, and fresh incubation medium (0.5 ml) was added containing TRH (100 nM) without or with the same additions as described above. Incubation was continued for 2 h at 37 C. This medium was removed, centrifuged (2000 × *g*), and stored at −20 C. TSH in incubation medium was measured by RIA (22).

### *Free fractions of iodothyronine derivatives*

The free fractions of iodothyronines, Tetrac and Triac, were determined by equilibrium dialysis (25). The free Triac fraction was corrected for the fact that around 70% of [$^{125}$I]Triac precipitated with $MgCl_2$. The free fractions of all iodothyronines used in this study were determined in incubation medium with 0.5% BSA; those of Tetrac and $T_4$ were also determined in incubation medium with 0.1% BSA. The free fractions of Triac and Tetrac were also determined in the serum of five control subjects and six NTI patients.

### *Agar gel electrophoresis*

The distribution of Triac and Tetrac among serum binding proteins was determined by agar gel electrophoresis (26). The sera (50 $\mu$l) were mixed with bromophenol blue (1 $\mu$l) and $^{125}$I-labeled Triac or Tetrac ($10^6$ cpm). Gels were run for 60 min at 70 mA/slide (~150 V) at 15 C. Successive slices of 2 mm gel were counted, and the binding proteins $T_4$-binding globulin (TBG), albumin, and $T_4$-binding prealbumin (TBPA) were identified using the bromophenol blue staining of albumin as a reference; the region between the origin and albumin contained TBG, whereas the region anodal to albumin contained TBPA.

### *Statistics*

The statistical significance of any of the tested compounds on [$^{125}$I]$T_3$ uptake or on TSH secretion was evaluated by Student's *t* test or one-way analysis of variance and Duncan's test for multiple comparisons. $P < 0.05$ was regarded as statistically significant.

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## Results

### *Free fractions of iodothyronine derivatives*

The free fractions of 3,3'-$T_2$, $T_3$, $rT_3$, $T_4$, Triac, and Tetrac in medium with 0.5% BSA are shown in Fig. 1. The free fraction decreases as the number of iodine atoms in the compound increases, whereas a change in the alanine side-chain to acetic acid, as in Triac and Tetrac, decreases the free fraction by a factor 8 (Triac *vs.* $T_3$) or 100 (Tetrac *vs.* $T_4$). The free fractions of Tetrac and $T_4$ in medium with 0.1% BSA amounted to 0.029 ± 0.001% (n = 3) and 3.33 ± 0.08% (n = 4), respectively, *i.e.* again a 100-fold difference. The free $T_3$ fraction (3.61 ± 0.05%; n = 12) did not change after the addition of 1 nM-1 μM $T_3$, Triac, Tetrac, or $rT_3$, but it increased to 4.53 ± 0.13% (n = 4), 4.51% (n = 2), 4.17 ± 0.02% (n = 4), and 4.43 ± 0.09% (n = 4) after the addition of 10 μM $T_3$, Triac, Tetrac and $rT_3$, respectively.

### *Time course of uptake of [$^{125}$I]$rT_3$ and [$^{125}$I]Tetrac*

Figure 2 shows the time course of [$^{125}$I]$rT_3$ uptake compared with that of [$^{125}$I]$T_3$ expressed as a percentage of the dose (Fig. 2A) or as femtomoles per pM free hormone (Fig. 2B). The uptake curve for [$^{125}$I]$T_3$ was similar to that previously described (11, 22), showed a steep phase up to 1 h of incubation, and reached equilibrium between 1–4 h of incubation (Fig. 2A). At any time point of the experiment, the uptake of [$^{125}$I]$rT_3$ was only 3–4% of the uptake of [$^{125}$I]$T_3$ (Fig. 2A, *lower curve*). When expressed as femtomoles per pM free hormone, there was still a large difference between the uptake of the two hormones, but as the free fraction of $rT_3$ was half that of $T_3$, the values for $rT_3$ uptake were about 10% of those for $T_3$ uptake (Fig. 2B).

Similar comparisons were made for the uptake of [$^{125}$I]Tetrac and [$^{125}$I]$T_4$ in incubation medium with 0.1% BSA (Fig. 3). Uptake of [$^{125}$I]$T_4$ showed a steep phase up to 1 h of incubation and reached a plateau at 4 h of incubation (Fig. 3A, *upper curve*). Uptake of [$^{125}$I]Tetrac, expressed as percent dose, was less than 0.15% at any time point of the incubation (Fig. 3A, *lower curve*), and [$^{125}$I]$T_4$ uptake was about 10-fold higher at equilibrium (Fig. 3A, *upper curve*). However, when the data were expressed as femtomoles per pM free hormone, the picture was precisely reversed; the maximum uptake of [$^{125}$I]Tetrac was 1.16 ± 0.16 (n = 6) fmol/pM free Tetrac, and

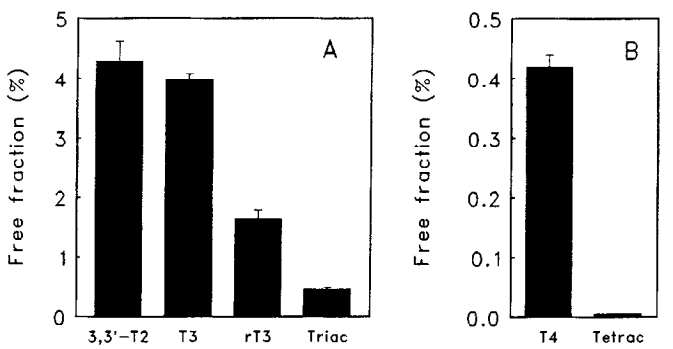


FIG. 1. Free fraction of iodothyronines and analogs in incubation medium with 0.5% BSA. *Bars* show the mean ± SE of three to eight determinations of the free fraction by equilibrium dialysis. A and B differ by a factor of 10 in the scale on the y-axis.

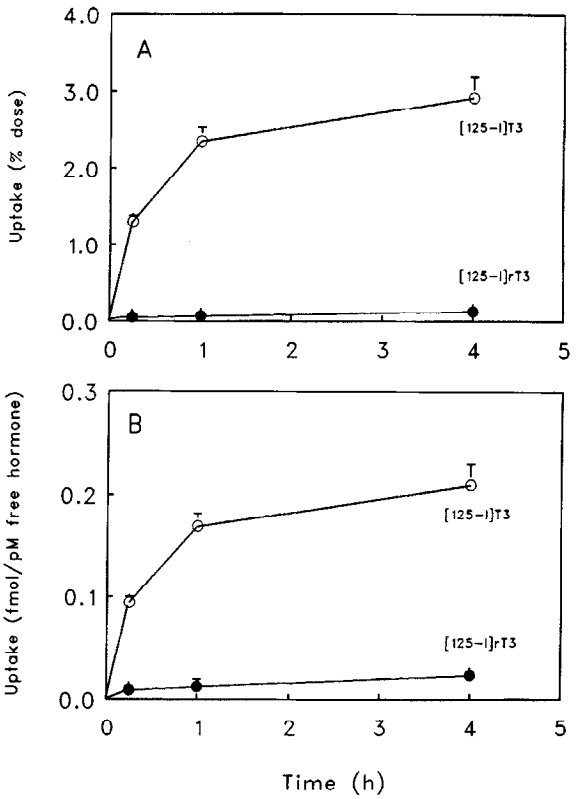


FIG. 2. Time course of uptake of [$^{125}$I]$T_3$ (○) and [$^{125}$I]$rT_3$ (●) in cultured anterior pituitary cells expressed as the percent dose (A) or as femtomoles per pM free hormone (B). Cells were cultured for 3 days at a density of 500,000 cells/well. After preincubation for 30 min in incubation medium with 0.5% BSA, cells were incubated for 15 min, 1 h, or 4 h in the presence of [$^{125}$I]$T_3$ (50,000 cpm) or [$^{125}$I]$rT_3$ (100,000 cpm). Data represent the mean ± SE of six observations from two independent experiments.

that of [$^{125}$I]$T_4$ was 0.15 ± 0.01 (n = 6) fmol/pM free $T_4$ ($P < 0.001$; Fig. 3B).

### *Metabolism of iodothyronines and Tetrac*

Table 1 shows the presence of iodide and conjugates in the incubation medium (*first two columns*) and HPLC analysis of the cell-associated radioactivity (*last four columns*) after incubation of pituitary cells with [$^{125}$I]$T_3$, [$^{125}$I]$T_4$, and [$^{125}$I]$rT_3$ for 24 h. After incubation with [$^{125}$I]$T_3$, no iodide was detectable with only a small amount of conjugates. Furthermore, almost all cell-associated radioactivity represented [$^{125}$I]$T_3$, indicating that $T_3$ is not metabolized in pituitary cells. Within the same experiment, however, [$^{125}$I]$T_4$ was metabolized, as shown by the production of iodide in the medium and the presence of [$^{125}$I]$T_3$ in the cells, representing 20% of the cell-associated radioactivity. The conversion of [$^{125}$I]$T_4$ to [$^{125}$I]$T_3$ was not inhibited by simultaneous incubation with 100 μM PTU, but was almost completely blocked by the presence of 1 μM unlabeled $T_4$. Finally, incubation with [$^{125}$I]$rT_3$ resulted in a significant production of iodide and conjugates, whereas about 6% of the cell-associated radioactivity represented [$^{125}$I]$T_2$.

The 24-h uptake and metabolism of [$^{125}$I]Tetrac were compared with those of [$^{125}$I]$T_4$ (Table 2). To ensure that uptake

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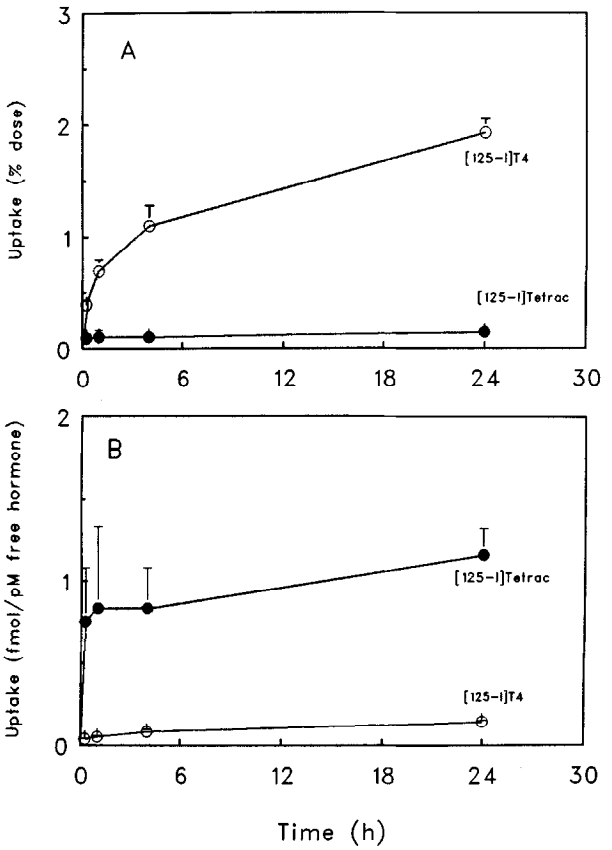


FIG. 3 Time course of uptake of [$^{125}$I]$T_4$ (○) and [$^{125}$I]Tetrac (●) in cultured anterior pituitary cells expressed as the percent dose (A) or as femtomoles per pM free hormone (B). Cells were cultured and the experiment was performed as described in Fig. 2, except that the incubation medium contained 0.1% BSA and [$^{125}$I]$T_4$ (0.1–2 × $10^6$ cpm) or [$^{125}$I]Tetrac (0.2–2 × $10^6$ cpm). Data represent the mean ± SE of six observations from two independent experiments.

of [$^{125}$I]Tetrac was large enough to allow Sephadex LH-20 chromatography and HPLC, the experiment was carried out with 0.01% and 0.1% BSA in the incubation medium. As shown in Table 2, about 15% of the cell-associated radioactivity of [$^{125}$I]$T_4$ and [$^{125}$I]Tetrac represented [$^{125}$I]$T_3$ and [$^{125}$I]Triac, respectively, after incubation for 24 h under the two experimental conditions. Net release of [$^{125}$I]iodide into the medium was: in 0.01% BSA, 3.9% (n = 2) from [$^{125}$I]$T_4$ and 1.8 ± 0.2% (n = 3) from [$^{125}$I]Tetrac; and in 0.1% BSA, 2.8 ± 0.3% (n = 3) from [$^{125}$I]$T_4$ and 0.6 ± 0.1% (n = 3) from [$^{125}$I]Tetrac.

### *Effects of $rT_3$, Tetrac, and Triac on [$^{125}$I]$T_3$ uptake*

Due to the very low tracer uptake (percent dose) of [$^{125}$I]Tetrac and [$^{125}$I]$rT_3$, it was impossible to perform detailed studies on the effects of thyroid hormone analogs or metabolic inhibitors on the plasma membrane uptake of these tracers. Therefore, we studied the effects of unlabeled Tetrac and Triac on the time course of [$^{125}$I]$T_3$ uptake (Fig. 4) as well as the effects of unlabeled $rT_3$ and Tetrac on the 15-min uptake of [$^{125}$I]$T_3$ (Fig. 5).

The uptake of [$^{125}$I]$T_3$ was at any time point significantly reduced by the presence of 10 μM Tetrac or Triac, and the effects of these analogs were similar. The effects of Tetrac and Triac were approximately 35%, 45%, and 70% after 15 min, 1 h, and 4 h of incubation, respectively (all $P < 0.001$; Fig. 4).

Measurements of [$^{125}$I]$T_3$ uptake in the linear part of the curve presented in Fig. 4, *i.e.* after 15 min of incubation, can be used to study uptake at the level of the pituitary plasma membrane (22, 23). The effects of Tetrac and $rT_3$ at concentrations from 1 nM to 1 μM on the 15-min uptake of [$^{125}$I]$T_3$ were compared with those of unlabeled $T_3$. The effect of unlabeled $T_3$ increased progressively with the concentration, reaching a maximum of 68% suppression ($P < 0.05$) at 10 μM. In contrast, Tetrac and $rT_3$ did not affect [$^{125}$I]$T_3$ uptake up to a concentration of 1 μM, but reduced [$^{125}$I]$T_3$ uptake by about 45% ($P < 0.05$) at a concentration of 10 μM (Fig. 5).

### *Effects on TRH-induced TSH release*

Table 3 shows the effects of Tetrac and $rT_3$ compared to those of Triac on TSH release during short term exposure to TRH. TSH release increased by approximately 90% after exposure to 100 nM TRH for 2 h ($P < 0.05$). This effect was significantly reduced by preincubation (2 h) and incubation with Triac at a concentration as low as 0.01 nM (71% suppression; $P < 0.05$) and was fully blocked at concentrations of 1–10 nM Triac and higher ($P < 0.05$). Preincubation and incubation with Tetrac resulted in a dose-dependent inhibition that was significant at 1 nM, and a maximum (82% inhibition; $P < 0.05$) was seen with a concentration of 1 μM. Preincubation and incubation with $rT_3$ showed a smaller inhibitory effect than that seen with Tetrac. Only at a $rT_3$ concentration of 1 μM was the TSH response to TRH significantly reduced (57%; $P < 0.05$). A direct comparison between Tetrac and $T_4$ in a single experiment showed that 100 nM Tetrac reduced TRH-induced TSH release to the same extent as 10 nM $T_4$ (36% and 27% inhibition, respectively; Table 4).

Similarly, the effects of preincubation and incubation with 3,3′-$T_2$ and 3,5-$T_2$ were compared with those of $T_3$ (Table 5). The stimulation during short term exposure to 100 nM TRH was 133% ($P < 0.05$) in these experiments. Preincubation and incubation with $T_3$ resulted in a dose-dependent inhibition of the TSH response to TRH, which was significant at concentrations of 1 nM and higher. 3,5-$T_2$ reduced the TSH response significantly at a dose of 1 nM, and the effect of 100 nM 3,5-$T_2$ was as great as that of 1 nM $T_3$ (55% suppression; $P < 0.05$). 3,3′-$T_2$ showed no inhibitory effect in doses less than 100 nM, but this dose had the same effect as 100 nM 3,5-$T_2$ or 1 nM $T_3$.

### *Serum binding and free fraction of Triac and Tetrac*

The binding of Triac and Tetrac to serum proteins was determined in four control subjects and four NTI patients (Table 6). In control subjects, Triac was equally bound to albumin and TBPA, but in NTI patients, more Triac was bound to albumin than to TBPA. Binding of Triac to TBG was negligible in both groups. In contrast, Tetrac was almost exclusively bound to TBPA in control subjects. Binding of Tetrac to albumin and TBG was about 5%. Also, the binding of Tetrac to TBPA was reduced in NTI patients, whereas

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**TABLE 1.** Metabolism of $[^{125}I]T_3$, $[^{125}I]rT_3$, and $[^{125}I]T_4$ in cultured anterior pituitary cells

| | Medium (%) | | Cellular content (%) | | | |
|---|---|---|---|---|---|---|
| | Iodide | Conjugates | $[^{125}I]T_3$ | $[^{125}I]T_4$ | $[^{125}I]rT_3$ | $[^{125}I]T_2$ |
| $[^{125}I]T_3$ (3) | 0.0 ± 0.0 | 0.28 ± 0.01 | 98.0 ± 0.1 | | | |
| $[^{125}I]T_4$ (6) | 1.10 ± 0.04 | 0.16 ± 0.01 | 21.0 ± 0.6 | 71.2 ± 0.3 | | |
| $[^{125}I]T_4$ + 100 μM PTU (3) | 1.89 ± 0.03[a] | 0.16 ± 0.0 | 21.2 ± 0.5 | 70.9 ± 0.9 | | |
| $[^{125}I]T_4$ + 1 μM $T_4$ (3) | 0.44 ± 0.09[a] | 0.32 ± 0.02[a] | 1.9 ± 0.3[a] | 88.1 ± 1.8[a] | | |
| $[^{125}I]rT_3$ (3) | 1.86 ± 0.15 | 2.12 ± 0.02 | | | 82.4 ± 1.0 | 6.3 ± 0.6 |

Data represent the mean ± SE of three to six observations in a single experiment as indicated in *parentheses*. Anterior pituitary cells were cultured for 3 days at a density of 500,000 cells/well. The cells were preincubated for 30 min in incubation medium containing 0.5% BSA. Then they were incubated for 24 h in the same medium containing $2 \times 10^6$ cpm of any of the tracers without or with 100 μM PTU or 1 μM $T_4$. After incubation, aliquots of the medium and the cells were analyzed by Sephadex LH-20 chromatography. The iodothyronine fraction from the cells was further analyzed by HPLC.

[a] $P < 0.001$ *vs.* $[^{125}I]T_4$ alone.

**TABLE 2.** Metabolism of $[^{125}I]T_4$ and $[^{125}I]$Tetrac in cultured anterior pituitary cells

| Substrate | Uptake (% dose) | $[^{125}I]$ activity in cells (%) | |
|---|---|---|---|
| | | $T_4$ or Tetrac | $T_3$ or Triac |
| 0.01 % BSA | | | |
| $[^{125}I]T_4$ | 5.69 ± 0.09 | 85.2 ± 0.8 | 12.0 ± 0.7 |
| $[^{125}I]$Tetrac | 0.35 ± 0.08 | 74.5 ± 3.6 | 15.2 ± 1.7 |
| 0.1% BSA | | | |
| $[^{125}I]T_4$ | 2.03 ± 0.07 | 73.1 ± 2.5 | 17.8 ± 1.2 |
| $[^{125}I]$Tetrac | 0.16 ± 0.01 | 79.4 ± 4.2 | 12.6 ± 0.4 |

Data represent the mean ± SE of three observations in a single experiment. Anterior pituitary cells were cultured for 3 days at a density of 500,000 cells/well. The experiment was performed as described in Table 2 with $2 \times 10^6$ cpm $[^{125}I]T_4$ or $[^{125}I]$Tetrac with BSA concentrations in the incubation medium as indicated. The product of $[^{125}I]T_4$ metabolism was identified by HPLC as $T_3$, and that of $[^{125}I]$Tetrac metabolism was identified as Triac.

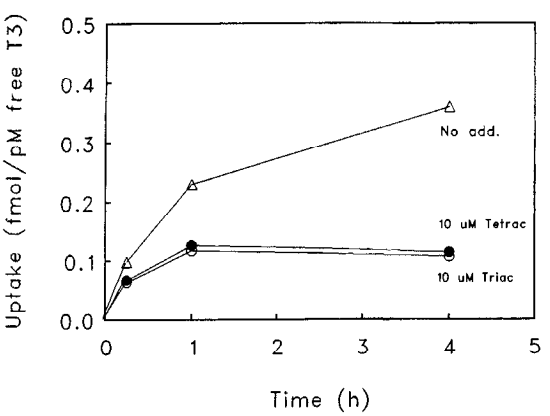


FIG. 4. Effects of unlabeled Triac or Tetrac on the uptake of $[^{125}I]T_3$ in cultured anterior pituitary cells. Cells were cultured and the experiment was performed as described in Fig. 2, except that the incubation contained $[^{125}I]T_3$ (50,000 cpm) (△) without or with 10 μM Triac (○) or Tetrac (●). Data represent the mean ± SE of three to six observations from two independent experiments.

more Tetrac was bound to albumin than in the control subjects (Table 6).

The changes in serum binding during NTI (Table 6) resulted in remarkable changes in the free fractions of Triac and Tetrac in NTI patients (Fig. 6). In control subjects (no. 1–5), the free Triac fraction was approximately 0.04%, whereas that of Tetrac was 0.0011%. Similarly low values for the free fractions of Triac and Tetrac in normal serum have previously been described (27). During NTI (patients 6–11), the

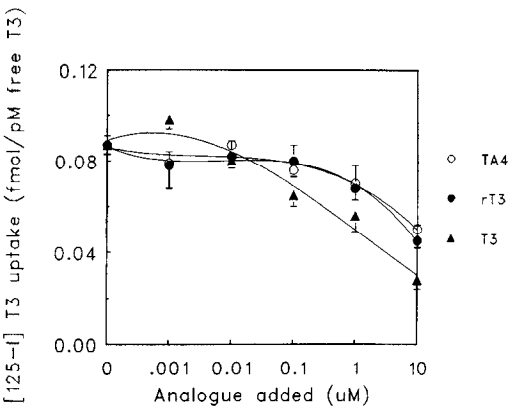


FIG. 5 Effects of unlabeled $T_3$ (▲), $rT_3$ (●), or Tetrac (○; $TA_4$) on the 15-min uptake of $[^{125}I]T_3$ in cultured anterior pituitary cells. Cells were cultured and uptake of $[^{125}I]T_3$ was measured for 15 min as described in Fig. 2. Incubation was performed with $[^{125}I]T_3$ (50,000 cpm) without or with unlabeled $T_3$, $rT_3$, or Tetrac present in concentrations of 1 nM to 10 μM. Each *point* represents the mean ± SE of six observations from two independent experiments.

free Triac fraction increased 2- to 8-fold, and that of Tetrac increased 2- to 12-fold.

## Discussion

The present study shows that both $[^{125}I]$Tetrac and $[^{125}I]rT_3$ are taken up and metabolized in cultured anterior pituitary cells. In addition, Tetrac and its major metabolite Triac significantly reduced the TSH response to TRH at total hormone concentrations of 1 and 0.01 nM, respectively, whereas $rT_3$ and its metabolite 3,3′-$T_2$ had to be added at total concentrations of 1 μM and 100 nM, respectively, to obtain a significant reduction of the TRH-induced TSH release.

Expressed as percent dose, the uptake of $[^{125}I]$Tetrac and $[^{125}I]rT_3$ was very low. That both tracers were taken up by pituitary cells was indicated by the 24-h incubation experiments. Around 15% of the cell-associated radioactivity was $[^{125}I]$Triac after incubation with $[^{125}I]$Tetrac, 6% of cell-associated radioactivity was $[^{125}I]T_2$ after incubation with $[^{125}I]rT_3$, and 15–20% of cell-associated radioactivity was $[^{125}I]T_3$ after incubation with $[^{125}I]T_4$. The deiodination of $[^{125}I]T_4$ was almost completely blocked by simultaneous incubation with 1 μM unlabeled $T_4$, but not by 100 μM PTU. $[^{125}I]T_3$ was not metabolized in pituitary cells. Together,

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**TABLE 3.** Effects of Tetrac, $rT_3$, and Triac on TSH release from anterior pituitary cells during short term exposure to TRH

| Exp conditions | TSH release (ng) | Stimulation (%) |
|---|---|---|
| Controls (23) | 3.15 ± 0.22 | |
| + 100 nM TRH (25) | 5.95 ± 0.14 | +89[a] |
| TRH + 1 nM Tetrac (3) | 4.87 ± 0.26 | +55[b] |
| TRH + 10 nM Tetrac (6) | 4.82 ± 0.32 | +53[b] |
| TRH + 100 nM Tetrac (3) | 4.09 ± 0.39 | +30[b] |
| TRH + 1 μM Tetrac (9) | 3.73 ± 0.19 | +18[b] |
| TRH + 1 nM $rT_3$ (3) | 5.22 ± 0.64 | +66 |
| TRH + 10 nM $rT_3$ (6) | 5.29 ± 0.18 | +68 |
| TRH + 100 nM $rT_3$ (3) | 4.93 ± 0.43 | +57 |
| TRH + 1 μM $rT_3$ (6) | 4.50 ± 0.11 | +43[b] |
| TRH + 0.01 nM Triac (3) | 4.06 ± 0.35 | +29[b] |
| TRH + 0.1 nM Triac (3) | 3.83 ± 0.25 | +22[b] |
| TRH + 1 nM Triac (3) | 3.40 ± 0.26 | +8[b] |
| TRH + 10 nM Triac (9) | 3.10 ± 0.19 | −2[b] |
| TRH + 100 nM Triac (6) | 3.43 ± 0.26 | +9[b] |
| TRH + 1 μM Triac (6) | 2.90 ± 0.17 | −8[b] |

Data indicate the mean of four experiments, with the number of observations in *parentheses*. Each experiment included at least two of the tested compounds at comparable concentrations. Anterior pituitary cells were cultured for 3 days at a density of 500,000 cells/well. The cells were preincubated for 2 h in the absence or presence of Tetrac, $rT_3$, or Triac at concentrations of 0.1 nM to 1 μM. Then, they were incubated with TRH (100 nM) for 2 h without or with the additions indicated. TSH was measured by RIA.

[a] $P < 0.05$ *vs.* controls.

[b] $P < 0.05$ *vs.* TRH alone.

**TABLE 4.** Comparison of the effects of Tetrac and $T_4$ on TSH release during short term exposure to TRH

| Exp conditions | TSH release (ng) | Stimulation (%) |
|---|---|---|
| Controls (6) | 2.50 ± 0.49 | |
| + 100 nM TRH (6) | 5.93 ± 0.30 | +137[a] |
| TRH + 100 nM Tetrac (3) | 4.09 ± 0.39 | +64[b] |
| TRH + 1 μM Tetrac (3) | 3.60 ± 0.41 | +44[b] |
| TRH + 10 nM $T_4$ (3) | 4.33 ± 0.48 | +73[b] |
| TRH + 100 nM $T_4$ (3) | 4.30 ± 0.19 | +72[b] |

Data indicate the mean ± SE of three to six observations in a single experiment as indicated in *parentheses*. Anterior pituitary cells were cultured at a density of 500,000 cells/well. The experiment was performed as described in Table 3.

[a] $P < 0.05$ *vs.* controls.

[b] $P < 0.05$ *vs.* TRH alone.

these results indicated the presence of type II deiodinase activity in the cultured anterior pituitary cells (19).

Because of the low tracer uptake of [$^{125}$I]$rT_3$ and [$^{125}$I]Tetrac, it was impossible to test the effects of metabolic inhibitors and thyroid hormone analogs on the transport process. Therefore, we tested the effects of $rT_3$ and Tetrac on the uptake of [$^{125}$I]$T_3$. Neither $rT_3$ nor Tetrac had an effect on the 15-min uptake of [$^{125}$I]$T_3$ when added at concentrations of 1–100 nM, and both showed a significant reduction of [$^{125}$I]$T_3$ uptake (45%) at a concentration of 1 μM. In a previous study, the presence of 10 μM $rT_3$ reduced the 15-min uptake of [$^{125}$I]$T_4$ by 32%, whereas 10 μM Tetrac had no effect (23). As $T_3$ and $T_4$ probably share the same transport mechanism in

**TABLE 5.** Comparison of the effects of 3,3′-$T_2$, 3,5-$T_2$, and $T_3$ on TSH release from cultured anterior pituitary cells during short term exposure to TRH

| Exp conditions | TSH release (ng) | Stimulation (%) |
|---|---|---|
| Controls (21) | 2.78 ± 0.41 | |
| + 100 nM TRH (21) | 6.48 ± 0.45 | +133[a] |
| TRH + 0.1 nM 3,3′-$T_2$ (6) | 5.45 ± 0.36 | +96 |
| TRH + 1 nM 3,3′-$T_2$ (9) | 4.84 ± 0.43 | +74 |
| TRH + 10 nM 3,3′-$T_2$ (9) | 5.52 ± 0.24 | +98 |
| TRH + 100 nM 3,3′-$T_2$ (9) | 3.98 ± 0.29 | +43[b] |
| TRH + 0.1 nM $T_3$ (5) | 6.27 ± 0.52 | +125 |
| TRH + 1 nM $T_3$ (9) | 4.04 ± 0.52 | +45[b] |
| TRH + 10 nM $T_3$ (8) | 3.49 ± 0.44 | +26[b] |
| TRH + 100 nM $T_3$ (9) | 3.10 ± 0.26 | +12[b] |
| TRH + 0.1 nM 3,5-$T_2$ (6) | 5.14 ± 0.35 | +85 |
| TRH + 1 nM 3,5-$T_2$ (9) | 4.52 ± 0.28 | +63[b] |
| TRH + 10 nM 3,5-$T_2$ (9) | 4.47 ± 0.26 | +61[b] |
| TRH + 100 nM 3,5-$T_2$ (9) | 4.03 ± 0.36 | +45[b] |

Data indicate the mean ± SE of three experiments, with the number of observations in *parentheses*. Anterior pituitary cells were cultured at a density of 500,000 cells/well. Experiments were performed as described in Table 3.

[a] $P < 0.05$ *vs.* controls.

[b] $P < 0.05$ *vs.* TRH alone.

the pituitary (23, 28), one might expect that uptakes of $T_3$ and $T_4$ are equally inhibited by analogs.

The results obtained in the present and our previous study (23) do not argue against the possibility that $rT_3$ is taken up by the same transporter involved with $T_3$ and $T_4$ uptake. The conclusion for Tetrac is more complicated. The uptake of Tetrac per picomolar concentration of free hormone (~1 fmol) was as high as that of Triac (11). Furthermore, 10 μM Tetrac reduced the uptake of [$^{125}$I]$T_3$ (15 min to 4 h) to the same extent as 10 μM Triac. In our study of [$^{125}$I]Triac uptake (11), it was concluded that at least part of Triac entered the pituitary by a mechanism different from the $T_3$ transporter. Together, the similarities between Tetrac and Triac suggest that Tetrac enters the pituitary in the same way as Triac, *i.e.* at least partly by another mechanism than the $T_3/T_4$ transporter.

Tetrac was effective in reducing the TSH response to TRH at a total concentration of 1 nM, *i.e.* at a free concentration of 0.05 pM. 100 nM Tetrac showed the same inhibitory effect as 10 nM $T_4$ or 0.01 nM Triac. Corrected for the difference in free fraction in medium with 0.5% BSA (Tetrac, 0.005%; $T_4$, 0.5%; Triac, 0.5%), 5 pM Tetrac was thus as effective as 50 pM $T_4$ or 0.05 pM Triac. In a direct comparison between Triac and $T_3$, it was estimated that Triac was 8 times more effective than $T_3$ in reducing the TSH response to TRH (11). Thus, the order of potency for reducing TSH release in pituitary cells seems to be Triac > $T_3$ > Tetrac > $T_4$.

The question remains of whether the effect of Tetrac or $T_4$ on TSH release during 4 h of incubation is due to a direct effect or to an effect of their respective metabolites Triac and $T_3$. In a previous study on the metabolism of [$^{125}$I]$T_4$, it was found that iodide production into the medium after 6 h of incubation was about 40% of that found after 24 h (23). Assuming a similar value for Tetrac, and that a proportion-

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**TABLE 6.** Distribution of Triac and Tetrac between serum proteins in control subjects and NTI patients

| Subject no. | Triac (%) | | | Tetrac (%) | | |
|---|---|---|---|---|---|---|
| | TBG | Albumin | TBPA | TBG | Albumin | TBPA |
| Control 1 | 2.9 | 43.3 | 53.0 | 1.8 | 5.9 | 88.7 |
| Control 2 | 0 | 49.9 | 48.6 | 2.4 | 6.1 | 89.2 |
| Control 3 | 0 | 48.6 | 49.6 | 3.6 | 4.9 | 89.2 |
| Control 4 | 0 | 45.5 | 53.3 | 7.7 | 5.6 | 85.6 |
| Mean ± SE | 0.7 ± 0.7 | 46.8 ± 1.5 | 51.1 ± 1.2 | 3.9 ± 1.3 | 5.6 ± 0.3 | 88.2 ± 0.9 |
| NTI 7 | 0 | 59.0 | 39.7 | 3.0 | 6.2 | 89.2 |
| NTI 8 | 0 | 54.1 | 44.8 | 4.3 | 9.2 | 85.7 |
| NTI 9 | 1.0 | 68.6 | 29.7 | 2.6 | 10.5 | 83.2 |
| NTI 11 | 2.0 | 64.3 | 33.3 | 3.8 | 10.5 | 81.2 |
| Mean ± SE | 0.8 ± 0.5 | 61.5 ± 3.2[a] | 36.9 ± 3.4[a] | 3.4 ± 0.4 | 9.1 ± 1.0[b] | 84.8 ± 1.7 |

Data present individual values and the mean ± SE of four healthy control subjects (no. 1–4) and four patients with NTI (no. 7–9, 11). The distribution of Triac and Tetrac among serum proteins was determined by agar gel electrophoresis.

[a] $P < 0.005$, NTI *vs.* control subjects.

[b] $P < 0.025$, NTI *vs.* control subjects.

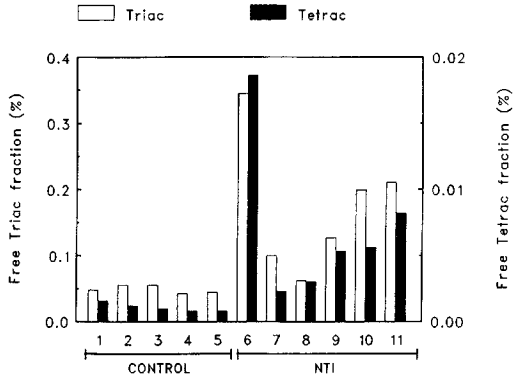


FIG. 6. Free fraction of Triac (□; *left* y-axis) and Tetrac (■; *right* y-axis) in serum obtained from five healthy control subjects (no. 1–5) and six patients with NTI (no.6–11). The free fractions were determined in duplicate by equilibrium analysis.

ally lower amount of [$^{125}$I]Triac is formed after 6 h of incubation (~5%; *i.e.* 40% of 15% [$^{125}$I]Triac), this would mean that 10 nM Tetrac (in a volume of 0.5 ml = 5 pmol) with an uptake of 0.2% results in 0.5 fmol intracellular Triac within 6 h. Furthermore, 0.1 nM Triac (in a volume of 0.5 ml = 50 fmol), with an uptake of 1% (11) would result in 0.5 fmol intracellular Triac. As the effect of 10 nM Tetrac on TRH-induced TSH release was similar to that of 0.1 nM Triac, the effect of Tetrac on TRH-induced TSH release can be the result of its deiodination to Triac.

$rT_3$ was only effective at a total concentration of 1 μM, whereas its metabolite 3,3′-$T_2$ induced a significant reduction in TRH-stimulated TSH release at a total concentration of 100 nM. In contrast, the $T_3$ metabolite 3,5-$T_2$, showed a significant effect on TSH release at a concentration of 1 nM and was about 100-fold less effective than $T_3$. Thus, 3,5-$T_2$ seems to be more effective in reducing TSH release than 3,3′-$T_2$, whereas the two diiodothyronines were equally effective in stimulation of the mitochondrial respiratory rate (16). However, our results support the *in vivo* observations in rats, in which 3,5-$T_2$ reduced TSH secretion at doses 10- to 100-fold higher than those of $T_3$ (18).

The question of whether $rT_3$ or its metabolite 3,3′-$T_2$ play a role in inhibition of TSH secretion during NTI can be addressed as follows: the normal total serum levels of $rT_3$ and 3,3′-$T_2$ are 0.40 and 0.11 nM (29). The same study showed that the serum total $rT_3$ concentration during NTI rose to 0.66 nM, whereas that of 3,3′-$T_2$ declined to 0.05 nM. This should be compared with the total hormone concentrations resulting in significant effects *in vitro*, *i.e.* 1 μM $rT_3$ and 100 nM 3,3′-$T_2$, respectively, in a medium containing 0.5% BSA where the free fraction will be at least 10-fold greater than that in serum. The difference between the effective concentrations *in vitro* and those present *in vivo* is thus at least a factor of 10,000, which is not in favor of any biological significance of the two compounds *in vivo*. The small inhibitory effect of $rT_3$ on TRH-induced TSH release cannot solely be explained by the low uptake of $rT_3$ by pituitary cells; compared with $T_3$, a 1000-fold higher total concentration of $rT_3$ was required to reduce TSH release to the same extent (1 μM $rT_3$ *vs.* 1 nM $T_3$). In terms of free concentration, the difference will be a factor of 500 (free $T_3$ fraction, 3.8%; free $rT_3$ fraction, 1.9%), whereas the difference in cellular uptake was a factor of 10.

The normal total serum Tetrac concentration varies between 0.7 nM (15) and 1.3 nM (13). With a free fraction of 0.0011%, the free Tetrac concentration in normal serum will be around 0.011 pM. As the production rate increases 2-fold in fasting (15) and the mean free fraction increases 7-fold during NTI, the resulting free concentration during fasting or NTI will be about 0.15 pM. The lowest Tetrac concentration tested that reduced the response to TRH significantly was 1 nM, *i.e.* a free Tetrac concentration of 0.05 pM, which is of same order of magnitude as the free concentrations occurring *in vivo*.

Just as postulated for Triac, the low serum Tetrac concentration may be the result of the very effective glucuronidation in the liver (30). Although the free concentration of Tetrac in serum (0.01–0.15 pM; see above) is lower than that of Triac (0.02–0.3 pM; estimated from Refs. 9, 11, and 31), $T_3$ (2–4 pM) (3), or $T_4$ (18–25 pM) (1–3), it is not excluded that Tetrac contributes to TSH suppression during NTI, because its uptake (per picomolar concentration of free hormone) into the pituitary is much larger than that of $T_3$ and $T_4$, whereas it is more effective than $T_4$ in reducing the TSH response to TRH. Also, the tentative conclusion that Tetrac (and Triac) are at least partly taken up in the pituitary by other transport mech-

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anisms than $T_3$ and $T_4$ would support a possible role of the compound *in vivo*.

The large rise in the estimated free concentrations of Tetrac and Triac in patients with NTI is not only due to the increase in the production rate (possibly 2- to 3-fold) (9, 15), but also to the rise in free fraction (2- to 12-fold; this study). The latter seems due to the fact that the level of TBPA, the most important binding protein for the two compounds in serum, strongly decreases during illness (10).

The conclusion of our study is that Tetrac and $rT_3$, the serum concentration of which increases during fasting and NTI, are taken up and metabolized in cultured anterior pituitary cells. Only Tetrac reduces the TSH response to TRH *in vitro* at concentrations comparable to those estimated *in vivo*. Because Tetrac probably enters the pituitary through another transport system such as that described for $T_3$ and $T_4$, our results do not exclude a potential role for Tetrac in the inappropriately low serum TSH observed during NTI.

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