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# Thyroid Hormonelike Actions of 3,3′,5′-l-Triiodothyronine and 3,3′-Diiodothyronine

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**Research Article**

l-Thyroxine is converted to 3,5,3′-l-triiodothyronine ($T_3$) as well as to 3,3′,5′-l-triiodothyronine (reverse $T_3$). One product of further deiodination is 3,3′-diiodothyronine (3,3′$T_2$). The serum levels of reverse $T_3$ and 3,3′$T_2$ change considerably in various physiological and disease states. We previously found that reverse $T_3$ and 3,3′$T_2$ bind to the solubilized hepatic nuclear "receptors" for thyroid hormones. This led us to study binding and actions of these metabolites in cultured rat pituitary cells in which glucose consumption and growth hormone production are regulated by $T_3$ and l-thyroxine.

Reverse $T_3$ and 3,3′$T_2$ stimulated growth hormone production and glucose consumption and inhibited nuclear binding of radioactive $T_3$. Either metabolite produced maximal effects that equaled those of $T_3$, and neither inhibited the $T_3$ response. Further, additive effects were observed when reverse $T_3$ was combined with submaximal concentrations of $T_3$.

In serum-free and serum-containing media, concentrations of 3,3′$T_2$ 50- to 70- and 10- to 100-fold greater, respectively, than those of $T_3$ were required for equivalent stimulations and for inhibition of nuclear binding by $T_3$. The relative activity differences under the two conditions can be attributed to weaker serum protein binding of 3,3′$T_2$ than $T_3$. With cells in serum-free media, reverse $T_3$ was a less avid competitor than 3,3′$T_2$ for $T_3$ binding by the nuclear receptors, and was less potent than 3,3′$T_2$ (0.001 the potency of $T_3$) […]



# Thyroid Hormonelike Actions of 3,3′,5′-L-Triiodothyronine and 3,3′-Diiodothyronine

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ABSTRACT L-Thyroxine is converted to 3,5,3′-L-triiodothyronine ($T_3$) as well as to 3,3′,5′-L-triiodothyronine (reverse $T_3$). One product of further deiodination is 3,3′-diiodothyronine (3,3′$T_2$). The serum levels of reverse $T_3$ and 3,3′$T_2$ change considerably in various physiological and disease states. We previously found that reverse $T_3$ and 3,3′$T_2$ bind to the solubilized hepatic nuclear "receptors" for thyroid hormones. This led us to study binding and actions of these metabolites in cultured rat pituitary cells in which glucose consumption and growth hormone production are regulated by $T_3$ and L-thyroxine.

Reverse $T_3$ and 3,3′$T_2$ stimulated growth hormone production and glucose consumption and inhibited nuclear binding of radioactive $T_3$. Either metabolite produced maximal effects that equaled those of $T_3$, and neither inhibited the $T_3$ response. Further, additive effects were observed when reverse $T_3$ was combined with submaximal concentrations of $T_3$.

In serum-free and serum-containing media, concentrations of 3,3′$T_2$ 50- to 70- and 10- to 100-fold greater, respectively, than those of $T_3$ were required for equivalent stimulations and for inhibition of nuclear binding by $T_3$. The relative activity differences under the two conditions can be attributed to weaker serum protein binding of 3,3′$T_2$ than $T_3$. With cells in serum-free media, reverse $T_3$ was a less avid competitor than 3,3′$T_2$ for $T_3$ binding by the nuclear receptors, and was less potent than 3,3′$T_2$ (0.001 the potency of $T_3$) in inducing growth hormone production or glucose oxidation. In incubations with serum-containing media, reverse $T_3$ was an ineffective competitor for $T_3$ binding, and had only 0.1 the inducing potency of 3,3′$T_2$ (0.001 the potency of $T_3$). The weaker activity of reverse $T_3$ relative to 3,3′$T_2$ in serum-containing media could be explained by stronger serum binding of reverse $T_3$ than 3,3′$T_2$. In addition, after long-term incubation of cells with radioactive reverse $T_3$, much of the cell-associated radioactivity was recovered as 3,3′$T_2$.

These studies suggest that reverse $T_3$ and 3,3′$T_2$ can stimulate thyroid hormone-regulated functions as weak agonists by acting via the same receptors that mediate $T_3$ actions. Moreover, some of the effects of reverse $T_3$ may be due to 3,3′$T_2$ produced by deiodination of reverse $T_3$.

## INTRODUCTION

Monodeiodination of thyroxine (3,5,3′,5′-tetraiodo-L-thyronine, $T_4$)[1] in peripheral tissues results in the production of 3,3′,5′-L-triiodothyronine (reverse $T_3$) as well as the biologically potent 3,5,3′-L-triiodothyronine ($T_3$, references 1–6). Studies in several laboratories have also shown that reverse $T_3$ is a normal component of human serum (5–11). Marked elevations of serum reverse $T_3$, as compared with

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[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; DIHPPA, 4-hydroxy-3,5-diiodophenylpyruvic acid; DIT, diiodothronine; $K_d$, apparent equilibrium dissociation constant; MEM, modified Eagle's minimal essential medium; MIHPPA, 4-hydroxy-3-iodophenylpyruvic acid; MIT, monoiodotyrosine; reverse $T_3$, 3,3′,5′-L-triiodothyronine; 3,3′$T_2$, 3,3′-diiodo-L-thyronine; $T_3$, 3,5,3′L-triiodothyronine; $T_4$, 3,5,3′, 5′-tetraiodo-L-thyronine.

normal adult levels, have been found in the cord blood of newborn humans (with high concentrations also present in amniotic fluid) and in adult serum in various disease states, including hepatic cirrhosis, chronic renal failure, acute febrile disease, and protein and calorie malnutrition (5–7, 9–11). Elevations of reverse $T_3$ are frequently accompanied by a decrease in serum $T_3$ (7, 10). 3,3′-diiodo-L-thyronine (3,3′$T_2$) is also a constituent of the serum iodothyronines, and its main source seems to be extrathyroidal (12). 3,3′$T_2$ is probably the major product of monodeiodination of reverse $T_3$ and may only be a minor product of $T_3$ metabolism (13).

These recent observations relating to reverse $T_3$ and 3,3′$T_2$ have directed attention to possible biological functions of these compounds. In earlier studies (14), reverse $T_3$ appeared to have antithyroid hormonelike properties with respect to regulation of calorigenesis. By contrast, Samuels et al. (15) found that reverse $T_3$ stimulated glucose consumption in cultured rat pituitary tumor cells ($GH_1$). However, these workers could not exclude the possibility that their results were owing to the presence of contaminating $T_3$. In some studies (16, 17), reverse $T_3$ was shown to possess as much as 5% the activity of $T_4$ in preventing the development of goiter in rats receiving propylthiouracil; in other studies (18), reverse $T_3$ was reported to have <3% of the $T_4$ goiter prevention or calorigenic action. In adult mouse hemopoietic cells, reverse $T_3$ had 200 and 100% the activity of $T_3$ and $T_4$, respectively, in potentiating the effect of erythropoietin (19). More recently, Chopra et al. (20, 21) reported that reverse $T_3$ had a weak effect (5.2% that of $T_4$) on the rat pituitary in suppressing the release of thyroid stimulating hormone in response to thyrotropin releasing factor. The potential actions of 3,3′$T_2$ have received less attention, and the available literature (16, 17, 22, 23) does not clearly define its intrinsic biological activity. In this discussion, we have excluded a review of actions of reverse $T_3$ and 3,3′$T_2$ in a number of potentially important systems in which the effects of $T_3$ and $T_4$ are observed only at hormone concentrations much greater than are achieved physiologically (24, 25).

We recently found that both reverse $T_3$ and 3,3′$T_2$ bind to the putative nuclear receptors for thyroid hormones in studies with solubilized preparations (26). These findings suggested that these substances could have intrinsic agonist or antagonist actions on thyroid hormone responses, and led us to study these metabolites in greater detail.

For the current studies we used cultured rat pituitary tumor cells ($GH_1$ line) that contain thyroid hormone receptors (27, 28) and respond to thyroid hormones by increases in glucose consumption (15) and growth hormone production (29), and by decreases in prolactin production (29). We also investigated another subline of these cells (GC) which differs from $GH_1$ cells in that the former divide more frequently (mean doubling time of 30 h as compared with 48 h for the $GH_1$ cells), produce more growth hormone, do not produce a significant amount of prolactin, and can be grown readily in spinner culture (30).

## METHODS

*Materials.* Reverse $T_3$ and 3,3′$T_2$ were kindly provided by Dr. Eugene Jorgensen of the University of California at San Francisco. The synthesis and analysis of these compounds have been reported previously (31, 32), and each migrated as a single peak in the thin-layer chromatography systems described earlier (26). Stock solutions were prepared in n-propanol at 1 mM. Exposure of the solutions to UV irradiation was avoided. $T_3$ was purchased from Sigma Chemical Co., (St. Louis, Mo.) and assayed for purity as previously described (26). Stock solutions were prepared in serum-free modified Eagle's minimal essential medium (MEM) Joklik (Grand Island Biological Co., Grand Island, N. Y.) at 5 μM. [$^{125}$I]Reverse $T_3$ (679 μCi/μg) was kindly provided by Dr. Ralph Cavalieri, also of the University of California at San Francisco, and was prepared by Abbott Laboratories, Diagnostics Div. (South Pasadena, Calif.) as described previously (6). Based on thin-layer chromatography in the solvent systems described previously (26), there was about 10% contamination of this material with $^{125}$I-iodide and minimal contamination with other species.

*Cell growth and incubation conditions.* Rat pituitary tumor cells of the $GH_1$ line, from the American Type Culture Collection (Rockville, Md.), were grown in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal calf serum. Cells of the GC subline (30), kindly provided by Dr. Carter Bancroft, Sloan-Kettering Institute for Cancer Research, New York, were grown in MEM Jorlik supplemented with 15% horse serum, 2.5% fetal calf serum, and 0.4 mM $CaCl_2$. Cells were grown at 37°C in monolayers in 75 $cm^2$ plastic flasks (Falcon Plastics, Oxnard, Calif.) in 5% $CO_2$. All media, sera, salt, and trypsin-EDTA solutions were obtained from Gibco and were prepared and sterilized in the Central Tissue Culture Facility of the University of California, San Francisco. For subculture, the medium was removed and each flask was incubated with 5 ml of a trypsin-EDTA solution (Grand Island Biological Co., no. 531) at 37°C for 10 min. Then, the trypsin-EDTA solution was removed and the cultures were resuspended in media. The detached cells were pelleted at 200 *g* for 10 min, and resuspended in fresh medium.

The examinations of responses to thyroid hormones and analogues were based on techniques previously described by Samuels and co-workers (15). Cells at the late logarithmic phase of growth were trypsinized and processed as described above. Approximately 55,000 cells/$cm^2$ were plated in a multiwell apparatus (Falcon Plastics). After incubation for 48–72 h under standard conditions, the medium was replaced by "hypothyroid media" containing 10% serum from a thyroidectomized calf (obtained from Rockland, Inc., Gilbertsville, Pa.) instead of horse and fetal calf serum, and incubated for 48–72 h to deplete the cells of $T_3$ present in the normal serum and to allow any thyroid-induced responses to subside. The $T_3$ and $T_4$ levels of the hypothyroid serum were determined; $T_3$ was measured by a radioimmunoassay (33) and $T_4$ by an adsorption technique

as described by the manufacturer (Abbott Laboratories). Both hormones were undetectable ($T_3 < 20$ ng/dl, $T_4 < 0.5$ μg/dl). The medium was then replaced with the dilutions of hormones and analogues in hypothyroid medium. The solvents used for the hormone were added to control incubations and did not produce detectable effects. After incubation for 48–60 h, the media of the $GH_1$ cell cultures were either assayed for glucose content immediately or frozen at −20°C and assayed the next day. With GC cell cultures after a 48 to 60-h incubation, the media were replaced with fresh media containing the hormones and analogues. After an additional incubation of 24 h the media were collected, frozen at −60°C, and assayed for growth hormone and glucose content within 1 wk. The freezing did not affect growth hormone or glucose levels. After removal of the media, both $GH_1$ and GC cultures were washed twice with 0°C phosphate-buffered saline (25 mM potassium phosphate, 0.1 M NaCl, pH 7.4) and were frozen at −20°C. After 2–7 days, cells were thawed, scraped in 0.5 ml of water, and portions were assayed for protein (34).

*Serum-free incubations.* $GH_1$ cells (80,000 cells/cm²) were plated and treated as described above up to the point of introducing the hormones and analogues to the cultures. At this step the media were removed and substituted with hormone dilutions prepared either in serum-free media or in serum-free media supplemented with 10% of a "serum substitute" whose composition and use in other cell culture systems has been previously described (35). All chemicals necessary for preparation of the serum substitute were obtained from Sigma Chemical Co. and Fisher Scientific Co. (Pittsburgh, Pa.). All hormones included in the composition of the serum substitute were excluded except insulin which was added to the medium (28 μU/ml; Eli Lilly and Co., Indianapolis, Ind.). After 48 h of incubation, the media were collected and 50-μl portions were diluted 1:5 or 1:10 in bovine serum albumin (BSA; Sigma Chemical) barbital buffer (1 mg/ml BSA, 0.05 M barbital, pH 8.6) and stored at −20°C. The cultures were washed twice with 1-ml portions for 0°C phosphate-buffered saline and stored at −20°C. After 2 wk the media were assayed for growth hormone and protein as described above.

*Glucose measurement.* Glucose was assayed by the glucose oxidase method in a Beckman glucose analyzer (Beckman Instruments Inc., Fullerton, Calif.). Glucose consumption was calculated by subtracting the glucose present in the media at the end of the incubation from that present initially and is presented in all cases as a percent of control incubations which did not receive any hormone.

*Growth hormone radioimmunoassay.* Antiserum to rat growth hormone (first antibody, prepared in Rhesus monkey) and rat growth hormone for radioiodination and for reference standard were provided by the rat pituitary hormone distribution program of the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health. Second antibody was a goat antimonkey gamma globulin, P4, obtained from Antibodies Incorporated (Davis, Calif.). Rhesus monkey carrier serum was obtained from Calbiochem (San Diego, Calif.).

For radioiodination, 10 μg of growth hormone (previously diluted in 0.01 M $NaHCO_3$ and stored at −20°C) was diluted in 10 μl of 0.3 M phosphate buffer pH 7.5 and iodinated with 2 mCi of $^{125}$I-iodide (carrier free; Amersham/Searle Corp., Arlington Heights, Ill.) in the presence of a stoichiometric amount (0.3 μg) of chloramine-T (Chester A. Baker Laboratories, Inc., Miami, Fla.). After 3 min, 25 μl (1 mg/ml) of $Na_2S_2O_5$ was added. After mixing, an excess of KI (75 μl, 10 mg/ml) and 5 ml of hypophysectomized dog plasma (obtained from the Department of Physiology, University of California, San Francisco) were added, and this reaction mixture was applied to a Sephadex G-100 column (1.5 × 24 cm), equilibrated in BSA-barbital buffer (0.5 M barbital, pH 8.6, 5 mg/ml of BSA. The peak of $^{125}$I-growth hormone eluted at 1.8 times the void volume of the column, well separated from aggregation products (in the void volume) and free iodide (in the included volume, 2.5 times the void volume). The $^{125}$I-growth hormone was diluted to a sp act of 400,000 cpm/ml with BSA-barbital buffer and then frozen at −60°C in 5- or 10-ml portions.

For the standard curve, duplicate 180-μl aliquots containing from 0.5 to 6 ng growth hormone were added to 120-μl aliquots consisting of a 1:1 mixture of $^{125}$I-growth hormone and first antibody (previously diluted 1:10,000 or 1:15,000 in BSA-barbital buffer). In all cases, the unknown samples were prepared and assayed in triplicate. After 4 days of incubation at 0–4°C, each tube received 240 μl second antibody solution (previously diluted 1:20 in BSA-barbital buffer) and 60 μl carrier serum (previously diluted 1:25 in BSA-barbital buffer). After overnight incubation at 0–4°C, the tubes were centrifuged at 1,000 *g* for 30 min, and radioactivity in the pellets was assayed in a gamma spectrometer (efficiency 74%). The quantity of growth hormone in the unknown samples was calculated after comparison with a standard curve.

Under the conditions described, the assay was sensitive between 0.5 and 5 ng. From 60–70% of the radioactivity in the tubes that did not contain any unlabeled hormone was precipitated under the conditions of the assay. The intra-assay variation (36) was 2.9%.

*Nuclear binding of $T_3$, reverse $T_3$ and 3,3′$T_2$.* Nuclear binding of $T_3$, reverse $T_3$, and 3,3′$T_2$ was examined in serum-free and serum-containing media in GC cells, according to techniques previously described by Samuels and Tsai (27). For serum-free experiments, GC cells in suspension culture ($5 \times 10^5$ cells/ml) were pelleted at 200 *g* for 5 min, resuspended in serum-free MEM Joklik ($5 \times 10^5$ cells/ml), and incubated at 37°C for 30 min to deplete the cells of endogenously receptor-bound hormone. Cells were then washed once in serum-free MEM Joklik and $3 \times 10^6$ cells in 2 ml of serum-free media were added to each of a series of tubes containing reverse $T_3$, 3,3′$T_2$, or $T_3$ freshly prepared in serum-free MEM Joklik (1 ml/tube). Radioactive [$^{125}$I]$T_3$ (sp act 502 μCi/μg, Amersham/Searle) to a final concentration of 30 pM was added immediately afterward. The tubes were gently agitated and incubated at 37°C for 2.5 h. During the incubation, the cells were gently resuspended every 30 min with a Pasteur pipette. After the incubation, the tubes were chilled on ice and 1 ml of 0°C phosphate-buffered saline was added to each tube. The tubes were then centrifuged at 800 *g* for 5 min. The supernate was discarded and the pellet was resuspended in 1 ml of Triton-magnesium buffer (Tris 50 nM, $MgCl_2$ 1 mM, Triton X-100 0.5%, pH 7.8). After 5 min the tubes were centrifuged for 8 min at 100 *g*. The Triton treatment was repeated and after the second centrifugation, radioactivity in the tubes was measured in a gamma spectrometer. Specific binding was calculated by subtracting from the total binding the nonspecific binding (cpm) observed in tubes that contained a 10,000-fold excess of nonradioactive $T_3$.

For the nuclear binding assays after incubations in serum-containing media, a modified technique was utilized. Duplicate tubes containing 0.3 ml of "hypothyroid serum" received radioactive [$^{125}$I]$T_3$ to yield a 0.2-nM concentration in the final reaction volume. Then the nonlabeled hormones (in 1 ml of serum-free MEM Joklik) were added. After equilibration for 3 h at 37°C, $3 \times 10^6$ cells, in 1.6 ml serum-free media (prepared as described above), were added

to each tube and the same procedure as for the serum-free binding assays was follows.

*$T_3$ and reverse $T_3$ radioimmunoassay.* $T_3$ radioimmunoassay was performed as described elsewhere (26). Reverse $T_3$ assays were performed by Ms. Margaret Hammond of the University of California at San Francisco and by Dr. Ralph Cavalieri by a double antibody technique (37). This method gives a cross-reactivity with $T_3$ of <0.01%, with 3,3'$T_2$ of 3.8%, and with $T_4$ of 0.062% (37).

*Metabolism of reverse $T_3$.* [$^{125}$I]Reverse $T_3$ was incubated with cells and media as described in the legend to Table I. After the incubations, the media were collected and cells were detached by trypsinization as described above. The cells were then centrifuged (300 *g* for 5 min) and the pellets were washed once with 3 ml of ice-cold phosphate-buffered saline and then frozen. After thawing, media samples were extracted with an equal volume of ethanol:butanol 1:3 (vol/vol) and the pellets (containing about $8 \times 10^6$ cells) were each extracted with 0.5 ml of the ethanol:butanol solution. After addition of the solvent, the mixtures were vigorously agitated and then centrifuged (5,000 *g*, 10 min), and samples of the organic phase were analyzed by thin-layer chromatography as described previously (26) using authentic reverse $T_3$ and 3,3'$T_2$ as internal standards.

## RESULTS

*Nuclear binding of $T_3$, reverse $T_3$, and 3,3'$T_2$ to GC cells.* To determine whether reverse $T_3$ or 3,3'$T_2$ could occupy the same nuclear receptor as $T_3$, we initially asked whether these metabolites could inhibit the binding of radioactive $T_3$ by the nuclear receptors in incubations with intact cells. As shown in Fig. 1, either metabolite could inhibit the binding of radioactive $T_3$ under serum-free conditions. $T_3$ is a more effective competitor; reverse $T_3$ and 3,3'$T_2$ have about 0.1 and 1.5%, respectively, the activity of $T_3$ (Fig. 1, Table I). The similarity of the activities of reverse $T_3$ and 3,3'$T_2$ relative to those obtained with the solubilized receptors (Table I) suggests that the inhibition is the result of competition with $T_3$ for binding rather than uptake. From these data we estimate apparent affinities (equilibrium dissociation constants; $K_d$) of these analogues for binding the receptors in the cells to be around 0.5, 70, and 20 nM for $T_3$, reverse $T_3$, and 3,3'$T_2$, respectively.[2]

[2] The apparent $K_d$ for $T_3$ was calculated from the relationship $K_d = C_{50} - T_3^*$. $K_d$ is the equilibrium dissociation constant. $C_{50}$ is the concentration of the competitor at which 50% inhibition of radioactive $T_3$ binding to the receptor is observed; $T_3^*$ is the concentration of radioactive [$^{125}$I]$T_3$ present in the reaction mixture. For these calculations, we assumed that in serum-free medium, the total $T_3$ equals the free $T_3$, because during the incubations <15% of the total is taken up by the cells. Also, the high media to cell radio further ensures that any $T_3$ that is endogenously bound is trivial in terms of the total $T_3$ concentration and therefore will not affect the measured $K_d$. For reverse $T_3$ and 3,3'$T_2$, the $K_d$ was calculated from the relationship $K_c = C_{50}K_d/(K_d + T_3^*)$, where $K_c$ is the apparent equilibrium dissociation constant for the competitor.

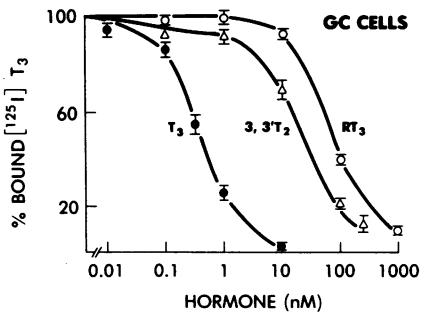


FIGURE 1 Inhibition of nuclear binding of [$^{125}$I]$T_3$ by nonradioactive $T_3$, reverse $T_3$ ($RT_3$), and 3,3'$T_2$ in GC cells and serum-free media. Shown is the mean and range determined from duplicate incubations of the specific binding. The abscissa shows the specific binding (see Methods) as a percent of that obtained in incubations without added competitor (6 fmol/$3 \times 10^6$ cells).

Different results were obtained when incubations were performed in serum-containing media (Fig. 2). 3,3'$T_2$ still exhibits competitor activity. By contrast, reverse $T_3$ is ineffective as a competitor, and in fact, at very high concentrations, even stimulates $T_3$ binding. As discussed later, the observed differences between serum-free and serum-containing conditions are likely explained by differences in the serum binding of the hormones.

*Actions of $T_3$, reverse $T_3$, and 3,3'$T_2$ on GC and $GH_1$ cells.* The influence of $T_3$, reverse $T_3$, and 3,3'$T_2$ in GC and $GH_1$ cells on glucose consumption in serum-containing medium is shown in Figs. 3–5 and in Table I. Both reverse $T_3$ and 3,3$T_2$ stimulate glucose consumption (Figs. 3–5). Concentrations of reverse $T_3$ 1,000-fold higher than $T_3$ are required to produce equivalent effects (Figs. 3–5), although concentrations of 3,3'$T_2$ one-hundredth that of reverse

TABLE I
*Receptor Binding and Biological Activity of Iodothyronines**

| | Activity (% of $T_3$) | | | |
|---|---|---|---|---|
| | $T_3$ | $T_4$ | Reverse $T_3$ | 3,3'$T_2$ |
| Binding by the nuclear receptors | | | | |
| Solubilized receptors (liver) | 100‡ | 20‡ | 0.2§ | 0.7‡ |
| Intact $GH_1$ cells | 100‖ | 11‖ | — | — |
| Intact GC cells | 100 | — | 0.5 | 1.8 |
| Biological effects | | | | |
| Growth hormone production | | | | |
| GC cells (serum containing) | 100 | — | 0.1–0.5 | — |
| Glucose consumption | | | | |
| GC cells (serum free) | 100 | | 0.06 | 1.5 |
| GC cells (serum containing) | 100 | — | 0.1–0.5 | 15 |

* In this table are summarized results obtained by us and others for nuclear binding and biological responses of $GH_1$ and GC cells to iodothyronines. Data expressed relative to $T_3$ (100%).
‡ Data from Latham et al. (26) and Jorgensen et al. (23).
§ Measured as previously described (26).
‖ Data from Samuels et al. (15, 27).

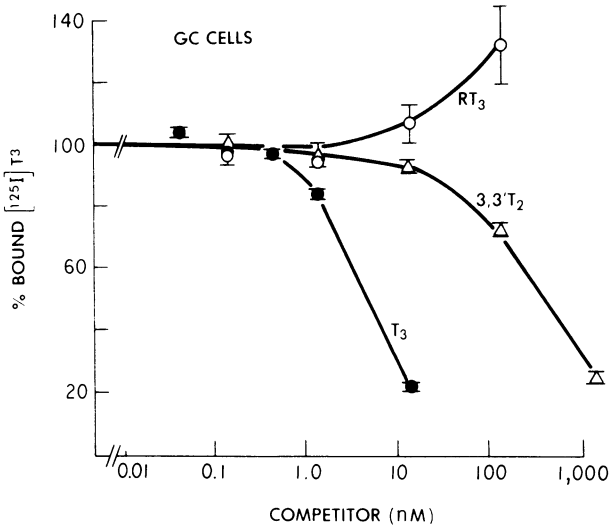


FIGURE 2 Specific nuclear binding of radioactive [$^{125}$I]$T_3$ in GC cells incubated in serum-containing media in the presence of nonradioactive $T_3$, reverse $T_3$ ($RT_3$), and 3,3'$T_2$. Shown are the means and ranges of values from duplicate incubations. (The abscissa shows the specific binding (see Methods) as a percent of that obtained in incubations without added competitor (10 fmol/3 × $10^6$ cells).

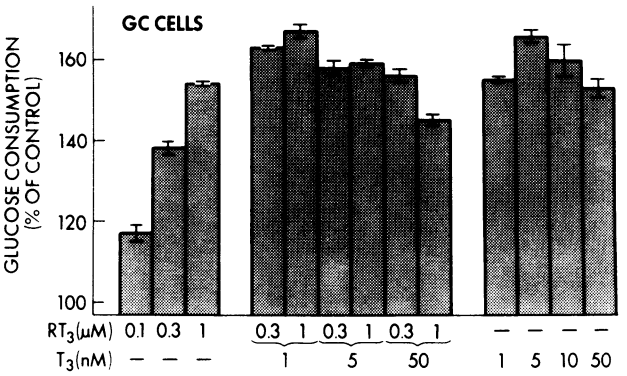


FIGURE 4 Effects of $T_3$ and reverse $T_3$ ($RT_3$) on glucose consumption by GC cells incubated with the indicated concentrations of $T_3$ or reverse $T_3$, alone or in combination. Shown are the means and ranges of duplicate determinations.

$T_3$ are required for a similar response (Fig. 3). Neither reverse $T_3$ or 3,3'$T_2$ was inhibitory, even at high concentrations (to 1 μM), and reverse $T_3$ and 3,3'$T_2$ produced maximal effects that approached but never exceeded those maximally obtainable by $T_3$. When reverse $T_3$ and $T_3$ were given together (Figs. 4 and 5), there was an augmentation in the responses at submaximal concentrations of the hormones (e.g., with 1 nM $T_3$ and 1 μM reverse $T_3$; Figs. 4–6; $P < 0.01$, Student's $t$ test (38)). However, the hormones in combination did not result in effects significantly greater than those elicited by maximally effective concentrations of $T_3$ alone. The data in Fig. 6 and Table I show that reverse $T_3$ can also stimulate growth hormone production by GC cells and also does not inhibit the effect of $T_3$.

The three analogues were also found to stimulate growth hormone production in experiments with serum-free media (Fig. 7 and Table I). The stimulations, especially with $T_3$ and reverse $T_3$, were obtained at analogue concentrations lower than required for equivalent stimulation in serum-containing media. The differences in the relative analogue potencies between the serum-free and serum-containing experiments can, in part, be explained by the varying binding of $T_3$, reverse $T_3$, and 3,3'$T_2$ to the serum proteins (39). Presumably, a higher proportion of the analogues

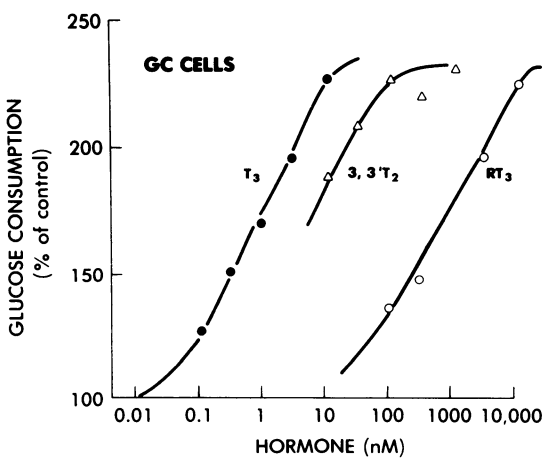


FIGURE 3 Effect of 3,3'$T_2$ on glucose consumption by GC cells incubated with the indicated hormone concentrations. Shown are the means and ranges of duplicate determinations. $RT_3$, reverse $T_3$.

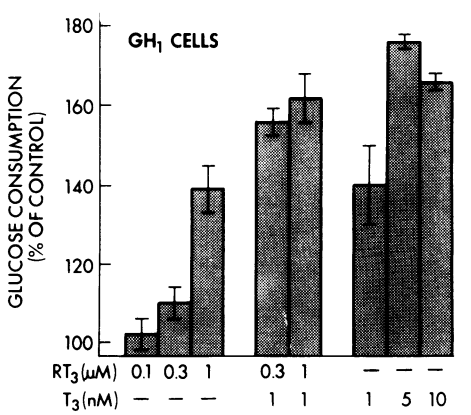


FIGURE 5 Effect of $T_3$ and reverse $T_3$ ($RT_3$) on glucose consumption by $GH_1$ cells incubated as described in Methods with the indicated concentrations of $T_3$ or reverse $T_3$, alone or in combination. Shown are the means and ranges from duplicate incubations.

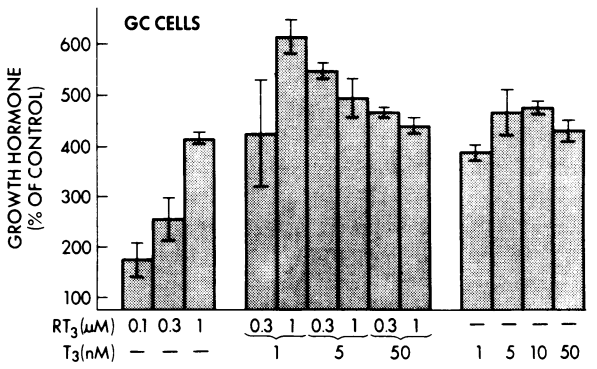


FIGURE 6 Effects of $T_3$ and reverse $T_3$ ($RT_3$) on growth hormone production by GC cells incubated with the indicated concentrations of $T_3$ or reverse $T_3$, alone or in combination. The results are expressed as a percent of control cultures which did not receive any hormones. Shown are the means and ranges from duplicate incubations. The rate of production of growth hormone in control cultures was 4.3 $\mu g/10^6$ cells per 24 h.

are free in serum-free media. This results in a shift in the dose-response curve to the left (Figs. 3 and 7). The concentration of free 3,3′$T_2$ is influenced to a lesser extent by the serum-proteins perhaps because of its lower affinity for them (39). Inasmuch as 3,3′$T_2$ is more potent than reverse $T_3$ and can result from deiodination of the latter, it was of interest to know whether or not this occurs in GH cells and could in part explain reverse $T_3$ action. We therefore studied the metabolism of reverse $T_3$.

*Metabolic conversion of reverse $T_3$ by the cells.* Cells were incubated with radioactive reverse $T_3$ in serum-containing media under conditions identical to those in which the measurements of the responses were performed. Radioactivity present in cells and media as reverse $T_3$ and 3,3′$T_2$ was then quantified utilizing the thin-layer system previously described (26; Table II). As shown, after a 72-h incubation of cells with radioactive reverse $T_3$, only small amounts (2–18% in various experiments) of the radioactivity associated with the cells was recovered as reverse $T_3$; more radioactivity migrated with 3,3′$T_2$. The reverse $T_3$ used is radioactively labeled in only one outer ring iodine. Because outer ring monodeiodination is random owing to free rotation of the ring around the ether bridge (40), radioactivity recovered as 3,3′$T_2$ would reflect only half the actual conversion of reverse $T_3$ to 3,3′$T_2$; half the deiodination would result in free $^{125}$I-iodide and nonradioactive 3,3′$T_2$ as products. Therefore, the actual amount of 3,3′$T_2$ present is double that of the measured radioactive 3,3′$T_2$. Thus, even though the media contained as much or more reverse $T_3$ than 3,3′$T_2$ at the end of the incubation, there was 5–16 times more cell-associated 3,3′$T_2$ than reverse $T_3$. These findings suggest that a substantial amount of the reverse $T_3$ which associates with the cells is converted to 3,3′$T_2$.

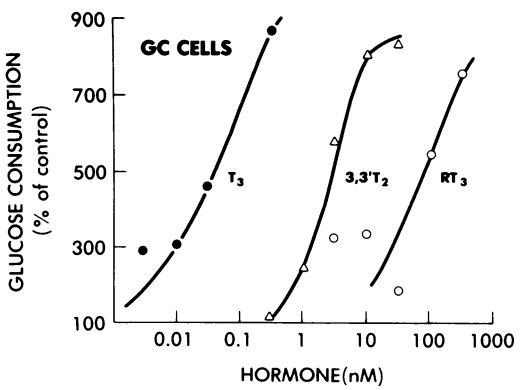


FIGURE 7 Effects of $T_3$, reverse $T_3$ ($RT_3$), and 3,3′$T_2$ on glucose consumption by GC cells in media with a serum substitute. Shown are the means (±SEM) from triplicate incubations from two experiments with serum-free and serum substitute media. The rate of production of growth hormone in control cultures was 1.6 $\mu g/10^6$ cells per 24 h.

TABLE II
*Conversion of Reverse $T_3$ to 3,3′$T_2$**

| Experimental conditions: Reverse $T_3$ | Recovery (%) | | |
|---|---|---|---|
| | | 3,3′$T_2$ | |
| | Reverse $T_3$ | Radioactive | Total |
| Added to media (no incubation | 78 (84, 72) | 2.5 (3, 2) | 5 |
| Incubated in media (no cells) | 74 (73, 75) | 2.5 (2, 3) | 5 |
| Incubated with media and GC cells | | | |
| Recovery in media | 42 (24, 60) | 20.5 (25, 15) | 41 |
| Recovery in cells | 4 (2, 6) | 33 (36, 30) | 66 |
| Incubated with media and $GH_1$ cells | | | |
| Recovery in media | 64 (59, 69) | 7.5 (4, 11) | 15 |
| Recovery in cells | 11 (4, 18) | 30 (33, 27) | 60 |

* 5 ml of "hypothyroid" media containing 1 $\mu$M nonradioactive reverse $T_3$ and $10^6$ cpm/ml radioactive reverse $T_3$ was introduced to 6-cm diameter plastic Petri dishes (Falcon Plastics) plus or minus cells ($10^5$/cm$^2$) and were incubated for 72 h. The dishes were then processed as described in Methods. Shown is the percent of the radioactivity applied to the thin-layer system that chromatographs with authentic reverse $T_3$ and 3,3′$T_2$. Most of the radioactivity that was not recovered in these two fractions was located at the solvent front. Shown are the means (and in parentheses the individual results) of determinations from two experiments, each performed with triplicate incubations. Replicate determinations within each experiment varied by <5%. The recovery of radioactive 3,3′$T_2$ has been doubled (total 3,3′$T_2$) to reflect the actual amounts present (see text).

Inasmuch as about 70% of the cell-associated radioactivity is accounted for by 3,3′$T_2$, reverse $T_3$, and iodide due to monodeiodination of reverse $T_3$ to 3,3′$T_2$, further deiodination products represent a minority of the thyronines associated with the cells. The data further suggest that the conversion of reverse $T_3$ to 3,3′$T_2$ is largely a cellular function, because there was little conversion of reverse $T_3$ to 3,3′$T_2$ when reverse $T_3$ was incubated for 3 days in media without cells (Table II).

## DISCUSSION

In the present studies we utilized rat pituitary tumor cells to study nuclear binding and biological actions of $T_3$, reverse $T_3$, and 3,3′$T_2$ as well as cellular metabolism of reverse $T_3$. We confirmed the findings of Samuels and co-workers (15) that $GH_1$ cells respond to physiological concentrations of $T_3$ by increased glucose consumption and growth hormone production. We also found that the GC line responds in a similar manner. In general agreement with Samuels' results (15, 29), the maximally effective $T_3$ concentration was in the range of 5 nM in serum-containing experiments, and $T_3$ effects were observed at lower concentrations of the hormone (e.g., 0.1 nM) in experiments with serum-free medium.

We also found that reverse $T_3$ and 3,3′$T_2$ stimulated glucose consumption and growth hormone production in both cell lines. Because the maximal responses elicited by either metabolite were as great as those of $T_3$, and because a small additive effect was observed when reverse $T_3$ was combined with submaximal concentrations of $T_3$, it appears that both analogues behave as thyroid hormone agonists rather than antagonists. These data also suggest that the measured biological effects of reverse $T_3$ and 3,3′$T_2$ are probably mediated via the same mechanism as are those of $T_3$.

The hypothesis that reverse $T_3$ and 3,3′$T_2$ actions are mediated by the intranuclear thyroid hormone receptor also receives some support from our previous studies (and those of Jorgensen and co-workers [23]), with the solubilized thyroid hormone receptors from liver in which reverse $T_3$ and 3,3′$T_2$ were shown to competitively inhibit binding of radioactive $T_3$ by the receptors. In the current studies, we found that reverse $T_3$ and 3,3′$T_2$ also compete with radioactive $T_3$ for binding to the nuclear sites in GC cells in experiments where incubations were performed using intact cells.

Inasmuch as these results could be obtained if the initial preparations of reverse $T_3$ and 3,3′$T_2$ were contaminated with $T_3$ or $T_4$, it is crucial to know that this is not the case. A number of considerations suggest that contaminations do not explain the results obtained. With respect to the reverse $T_3$ sample, radioimmunoassay showed no $T_3$ immunoreactive material under conditions in which 0.1 nM $T_3$ is readily detectable. Second, the technique of Block (32) excludes such contaminants. Even with the method of Shiba and Cahnmann (31),[3] it is extremely unlikely that the material was contaminated by as much as even 1% $T_3$.

$T_4$ also does not appear to be present in the reverse $T_3$ preparations, because the 25% contamination required to explain the previous receptor binding data (26) would have been detected by our thin-layer chromatography analysis as described previously (26). This level of contamination is also excluded by the elemental analysis of the material (31). Although $T_4$ radioimmunoassay might further clarify this issue, the antibodies to $T_4$ do significantly cross-react with the reverse $T_3$ preparations (26) so that it is impossible to draw any conclusions with this technique.

With 3,3′$T_2$, contaminations by 1% $T_3$ could explain the serum-free binding data and the response, and 10% contamination could explain the responses in serum-containing media. Likewise, a much greater contamination with $T_4$ would be required to explain the responses in serum-containing media. Again, from information related to the 3,3′$T_2$ source,[4] contamination appears to be extremely unlikely. If the observed results were owing to $T_3$ contamination, then the activity of the preparations relative to $T_3$ should be identical in experiments with serum-free and serum-containing medium. This is not the case. For example, the activity of 3,3′$T_2$ relative to $T_3$ is only 1.5% in experiments with serum-free medium (Fig. 1), whereas it is 10% in the experiments with serum-containing medium. Finally, a 10% contamination with $T_4$ would

[3] The reverse $T_3$ was synthesized by condensation of 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA) and monoiodotyrosine (MIT; 31). In this reaction the keto acid furnishes the phenolic ring and the amino acid furnishes the nonphenolic ring and the aliphatic chain of the iodothyronine (31). This method of preparation excludes possible $T_3$ contamination, unless there was significant contamination in the starting materials with both 4-hydroxy-3-iodophenyl-pyruvic acid (MIHPPA) and diiodothyronine (DIT). Whereas the reaction of DIHPPA and MIT results in an approximate 17–20% yield, MIHPPA and DIT react with a yield of only 2% (31). Thus, to obtain a 0.2–1% contamination of reverse $T_3$ with $T_3$ in the initial preparation, both DIHPPA and MIT would have to be contaminated by 10–50% with MIHPPA and DIT, respectively. This is highly unlikely, and such contamination would have been detected by the authors' chromatography or elemental analysis of the starting material (31).

[4] The 3,3′œ″≥₋₂ was prepared by condensation of iodo-L-tyrosine and an anisyliodonium salt that resulted in 3-iodo-L-thyronine. Further iodination yielded a mixture of iodothyronines, and 3,3′$T_2$ was isolated by thin-layer chromatography (41). The differences in $R_f$ between 3,3′$T_2$ and $T_3$ (0.22 vs. 0.4) in the solvent system used by the authors, makes $T_3$ contamination unlikely (41).

have been readily detected by our thin-layer chromatography, and cannot explain the biological responses of 3,3′$T_2$, because the relative potency of the preparation is greater in serum-containing than in serum-free media (Figs. 3 and 7). Thus, we consider it to be unlikely that the results were the result of contamination of either reverse $T_3$ or 3,3′$T_2$ preparations with either $T_3$ or $T_4$. Some earlier preparations have recently been shown by high pressure gas-liquid chromatography to contain unidentified contaminants.[5] These impurities are not $T_3$ or $T_4$ and were not detected in the preparations used in our present experiments. The presence of contaminants could explain why, in previous studies (26), we found a higher affinity for the solubilized receptors than was found in the present studies for receptors in either intact cells or solubilized preparations (Table I).

We found that the biological activity and the binding of 3,3′$T_2$ relative to $T_3$ was less in experiments in which serum-free medium was used than in experiments in which serum-containing medium was used. These differences could be explained if 3,3′$T_2$ does not bind as avidly to serum as $T_3$; this was found to be the case with human thyroxine-binding globulin (39), but was not directly studied in our situation for calf serum. Thus, a greater proportion of the circulating 3,3′$T_2$ could be available for receptor binding and hormone action than $T_3$ or $T_4$.

The quantitative aspects of reverse $T_3$ action were found to be more complex. Reverse $T_3$ readily inhibited $T_3$ binding in incubations with serum-free medium, but in fact stimulated binding of radioactive $T_3$ in incubations with serum-containing medium. These data may be explained if reverse $T_3$ binds more avidly to serum but less avidly to the receptor than $T_3$. It is known that human serum binds reverse $T_3$ more avidly than $T_3$ (39). Thus, more avid displacement of serum-bound than receptor-bound radioactive $T_3$ by reverse $T_3$ may result in more free radioactive $T_3$ available for receptor binding. These findings raise the conceivable possibility that reverse $T_3$ might act in our experiments or in physiological circumstances by displacing $T_3$ from serum. Although this could happen in conditions where substantial $T_3$ is present (e.g., in the binding experiment where 0.2 nM $T_3$ was added), there was no detectable contaminating $T_3$ present in the serum used in the experiments to elicit reverse $T_3$ effects. In any event, the experiments in which reverse $T_3$ was exposed to cells in serum-free media most clearly demonstrate the intrinsic activity of the latter.

Avid plasma binding can account for the greater biological activity of reverse $T_3$ in serum-free as compared to serum-containing medium. Furthermore, the biological activity of reverse $T_3$ relative to $T_3$ and 3,3′$T_2$ in general parallels its relative affinity for the intranuclear receptors. However, we found also that there was major cellular conversion of reverse $T_3$ to 3,3′$T_2$. In fact, only 12–18% of the cell-associated radioactivity was recovered as reverse $T_3$; most of the hormone (60–66%) was recovered as 3,3′$T_2$. By contrast, there was very little deiodination of reverse $T_3$ in incubations with media only. Therefore, it seems that in the intracellular environment, most of the reverse $T_3$ is converted to 3,3′$T_2$. Consequently, 3,3′$T_2$ may be a major form of the hormone in the cell available for receptor occupancy. In certain respects, then, reverse $T_3$ may act as a prohormone being converted to 3,3′$T_2$ intracellularly. These data also indicate that most of the cell-associated 3,3′$T_2$ can remain in an unmetabolized form for an appreciable time period.

To quantify precisely the contribution of 3,3′$T_2$ to reverse $T_3$ action would require a direct measurement of radioactive reverse $T_3$ and 3,3′$T_2$ on the receptor under the exact conditions in which the inductions are performed. This is difficult in practice because the affinity of these analogues for the receptor is much lower than that of $T_3$. Thus, there would be greater dissociation of these analogues during the isolation of nuclei, and more importantly, the higher concentrations of analogue required to produce adequate saturation of the receptors results in unacceptably high levels of nonspecific binding which masks an ability to directly observe specific binding.

These results, taken together, suggest that reverse $T_3$ and 3,3′$T_2$ can interact with the intranuclear receptors for thyroid hormone and influence thyroid hormone-mediated functions as agonists. The results also indicate that the quantitative aspects are complex and are influenced by serum binding and intracellular metabolism. Because thyroxine-binding globulin levels may vary, because target cells may vary in the extent to which reverse $T_3$ is metabolized, and because there may be intratissue variations in the affinity of $T_3$ and analogues for the receptors, caution must be excercised in transferring the quantitative aspects of the current studies to physiological situations. Nevertheless, it may be useful, for perspective and development of testable hypotheses, to use the available data, to ask to what extent could reverse $T_3$ and 3,3′$T_2$ influence thyroid hormone-regulated functions in the various states in which these hormones have been measured. Inasmuch as serum shifts the response to higher hormone concentrations, levels of 3,3′$T_2$ or reverse $T_3$ required for responses in vivo would probably be higher than those required in the current studies, in which 10% or no serum was present in the incubations. Levels of 3,3$T_2$

[5] Jorgensen, E. C. Personal communication.

have been measured in amniotic fluid and in the serum of normal adults, in cord blood of the new born, and in patients with hypothyroidism, hyperthyroidism, and cirrhosis (42, 43). Based on the current studies, the levels of 3,3′$T_2$ in these situations are probably too low (0.1–0.6 nM) for more than minimal thyroid hormone agonist activity to be attributed to this compound. In the adult, in conditions of excess reverse $T_3$ (e.g., cirrhosis, acute febrile disease, etc. [5–11]), serum levels of the hormone rarely exceed 75 ng/dt (1.2 nM), although levels to 300 ng/dl have been detected in the human fetus (5). The current studies show that even in undiluted plasma, reverse $T_3$ is essentially inactive at this concentration. In amniotic fluid, reverse $T_3$ levels may exceed 300 ng/dl (~5 nM). In this circumstance, reverse $T_3$ may be mostly free, and therefore its levels may approach 5 nM in cellular compartments. At 5 nM (Fig. 1), reverse $T_3$ can minimally influence $T_3$ binding to the receptors. Although the data raise the possibility that under certain circumstances, reverse $T_3$ and 3,3′$T_2$ may be active hormones, it is likely that these two hormones are mostly of minor importance in eliciting physiological responses of thyroid hormones.

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