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Adrenal gland 5′deiodinase activity (AG-5′D). Kinetic characterization and fractional turnover rate (FTr)

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We determined the kinetic parameters as well as the fractional turnover rate (FTr) and half-life ($t_{1/2}$) of rat adrenal gland 5′deiodinase activity (AG-5′D). Adrenal glands from male euthyroid or surgically thyroidectomized (Tx) Wistar rats were homogenized (HEPES, 10 mM; pH 7.5; sucrose, 0.25 M; EDTA 1 mM) and centrifuged at 10,000 *g* for 15 min at 4°C. The resulting crude microsomal supernatants were used for all measurements of 5′D activity. Using rT_3 (2–500 nM) the true Km and the Vmax values were of 20.2 nM and 289 fmol of I^- release/mg protein/h. With T_4 as substrate these values were 5.8 nM and 622 fmol/h/mg protein. Protein inhibitor (cycloheximide 6 mg/100 g wt) administration allowed to determine an FTr of 0.68 h^{-1} and a $t_{1/2}$ of 1.01 h. Results demonstrate that the greatest 5′D activity in the rat adrenal gland corresponds to isotype II, because the reaction is GTG and PTU-resistant (70–80%), accepts T_4 as a far better substrate than rT_3 (17-fold) and the former thyronine has a 50–90% inhibitory concentration in the 4–100 nM range. Furthermore, rats thyroidectomized for 5 and 15 days showed a conspicuous increase in cerebral cortex and adrenal 5′D-II activity. These characteristics as well as the rapid FTr and short $t_{1/2}$ are shared by type II 5′D present in rat pineal, pituitary and brain.

Keywords: adrenal gland; 5′deiodinase; kinetic parameters; fractional turnover rate; half-life

Introduction

The major iodothyronine secreted by the thyroid gland, thyroxine (T_4), is a prohormone whose deiodination by peripheral target tissues determines the local intracellular concentration of bioactive or inactive thyroid hormone. Deiodination of the T_4-outer ring, or so-called 5′-deiodination (5′D), constitutes the activating pathway which is catalyzed by two different enzymes known as 5′D-I and 5′D-II. On the contrary, removal of iodine from the inner ring of the molecule which is designated as 5-deiodination (5 D), constitutes the inactivating route and is catalyzed by the enzyme known as 5 D-III (Leonard & Visser, 1986; Kohrle *et al.*, 1987; Rosenberg, 1991). 5′D-I activity is higher in liver, kidney and the thyroid gland, than in the skeletal muscle, heart, spleen, lung, intestine and lactating mammary gland among organs. The activity of this enzyme varies in direct proportion to the thyroid status of the organism (Leonard & Visser, 1986; Kohrle *et al.*, 1987; Rosenberg, 1991). The 5′D-I is a selenoprotein selectively inhibited by 6-n-propyl-2-thiouracil (PTU) and gold-thioglucose (GTG). Recent translating studies using liver cDNA or native mRNA, have shown that 5′D-I activity is expressed in a single protein entity which, depending on the assay conditions, exhibits either 'low' (nanomolar) or 'high' (micromolar) apparent Km values for rT_3 and T_4 (Berry *et al.*, 1991; St Germain & Croteau, 1989). In contrast, 5′D-II activity is relatively insensitive to inhibition by PTU and GTG, and its apparent Km values for T_4 and T_3 range in the nanomolar interval (Safran & Leonard, 1991). The enzyme has been identified in several organs of neuroectoblastic lineage; e.g., central nervous system (Visser *et al.*, 1982), anterior pituitary (Kaplan, 1980), pineal and Harder glands (Guerrero & Reiter, 1992; Guerrero *et al.*, 1987), and its activity varies inversely to thyroid hormone supply (Leonard *et al.*, 1984). Because of our recent finding on this enzyme activity in the adrenal gland (AG) and its immediate increase during acute cold exposure in the rat (Anguiano *et al.*, 1991), we presently report the kinetic parameters, as well as the fractional turnover rate (FTr) and biological half-life ($t_{1/2}$) of rat-AG 5′D activity. This information is critical to any study aimed to understand the physiological regulation of thyronine deiodination.

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Results

Protein concentration, incubation time and pH

AG-5′D activity was a linear and dependent function of both the Mc-protein concentration (50–400 μg) and incubation time (Figures 1 and 2). Maximal enzyme activity was obtained at pH 7.5. In all subsequent experiments incubation time and pH were 3 h and 7.5, respectively. Compared to the fresh fraction, the Mc-fraction stored at −70°C retained its enzyme activity for one month (Figure 1).

Kinetic parameters

Table 1 summarizes the true Michaelis constants for both substrates (rT_3 and T_4) and cofactor in the Mc-fraction from the whole gland as well as from the cortex and medulla. Figure 3 depicts the kinetics of total rT_3–5′D activity as a

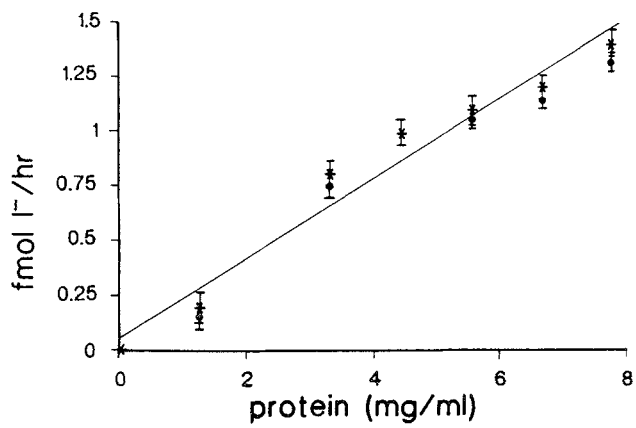

Figure 1 AG-5′D activity and protein concentration. Independently of the Mc fraction assayed, rT_3 5′-deiodination rate (fmol I released /hr) was proportional to the amount of protein present in the assay * freshly prepared or ● frozen (one month). Data represent the mean ± S.E. from 3 separate experiments performed in duplicate. Assay conditions were: 2 nM rT3; 20 mM DTT; pH 7.5; incubation time 3 h at 37°C

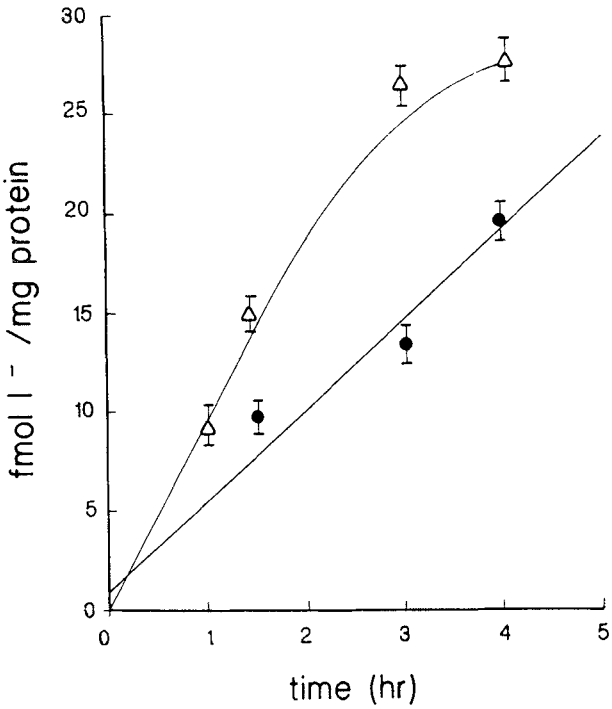

Figure 2 AG-5'D as a function of time and protein concentration. Two different protein concentrations (Mc) were tested (● 150 and Δ 250 μg/tube). Assay conditions were as in Figure 1, and each point represents the mean ± S.E. from three separate experiments performed in duplicate. With the lower protein concentration (150 μg) total activity was linear during the entire period of analysis (4 h). The activity is expressed as fmol I released/mg protein

function of substrate concentration. The enzyme was saturated by rT_3 concentrations ~100 nM. The inset in Figure 3 shows a double reciprocal plot (Lineweaver-Burk plot), from which the apparent kinetic constant values were: Km 18.3 nM and Vmax 272 fmol I/mg/h for rT_3. As shown in Figure 4a, in the presence of varying concentrations of DTT as second substrate, total rT_3–5'D activity increased as a function of dose, reaching maximal activity between 10–20 mM of DTT. A replot of the vertical axis intercept against the reciprocal of cofactor concentration was linear (shown in the inset of Figure 4a) and allowed the calculation of the true Km and Vmax values (20.2 nM and 289 fmol I/mg/h) for the whole adrenal gland. Figure 4b shows the primary Lineweaver-Burk plots of deiodination rate vs. DTT concentration and the inset is the replot of Y-axis intercepts as a function of 1/S. With this plot we determined the true Michaelis constants for DTT. The kinetic parameters for T4 5'D activity using Mc fraction from cortex and medulla (Table 1) were assessed in a series of similar experiments in

Table 1 Kinetic parameters of AG-5'-D

Organ	*Substrate*	*Vmax* (fmol I/mg/hr)	K_m	*Catalytic efficiency Vmax/Km rate*
Whole AG	rT3	289.0	20.2 nM	14
Cofactor				
Whole AG	DTT	263	7.3 mM	–
Whole AG	T_4	622	5.8 nM	107
Cofactor				
	DTT	628	6.06 mM	
Cortex	T_4	591	4.49 nM	131
Medulla	T_4	549	4.44 nM	123

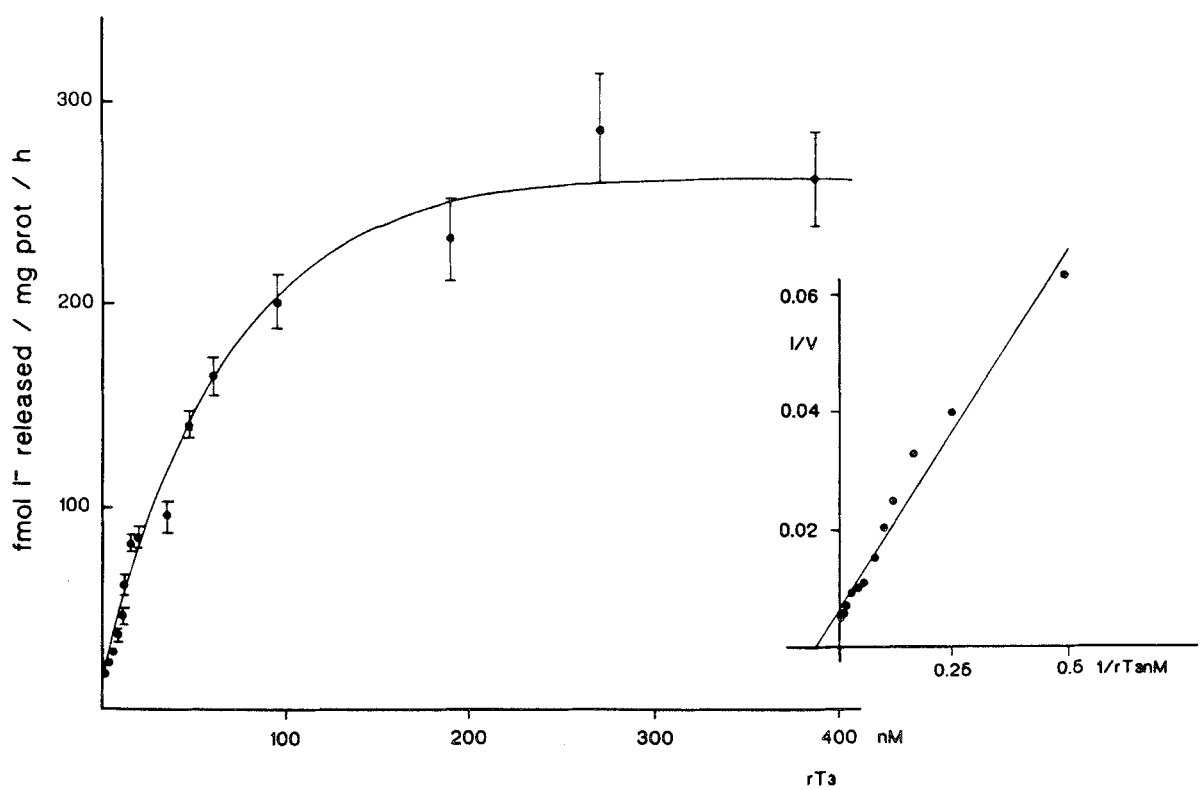

Figure 3 AG-5'D reaction kinetics. Enzyme activity is plotted as a function of substrate (rT_3) concentration. *Inset*: Lineweaver-Burk plot of the same results. *Assay Conditions*: protein 150 μg; substrate [rT_3] isotopic mixture (^{125}I-rT_3, 4 nM/rT_3, 2–400 nM); DTT 20 mM; pH 7.5 at 37°C for 3 h. Each point represents the mean ± S.E. of three independent experiments. The activity showed a saturable pattern. The Michaelis-Menten constants obtained were Km = 18.7 nM and Vmax = 272 fmol I/h/mg protein

which 1 mM PTU was present. Figure 5 shows the double reciprocal plot from these experiments.

Effects of T_4, PTU and GTG on 5'D activity

rT_3-5'D activity was reduced from 50 to 90% in the presence of increasing concentrations of unlabeled T_4 (4–100 nM), thus suggesting the preference of this substrate over rT_3. In contrast to this clear-cut competitive inhibition by the substrate, the presence of 10 mM PTU exhibited a moderate inhibitory effect (30%) on total AG-5'D activity. Moreover, when evaluating the effect of (2.5–10 mM) PTU on separate cortex and medulla Mc-fractions, dissimilar sensitivity was observed, PTU inhibited only 15% in medullar activity, whereas in cortex, the activity inhibition was 30% (Figure 6). The *in vitro* effect of GTG (0.01, 0.10, 1.0 and 10.0 μM) on rT_3 5'D-II activity in whole gland was also analysed. Results demonstrate that enzyme activity remains unaffected at GTG 0.01 and 0.10 μM whereas at 1.0 and 10.0 μM the activity is inhibited by 30 and 90% respectively).

Effect of thyroid status on AG-5'D-II

As shown in Figure 7, AG and cerebral cortex 5'D-II activity from surgically thyroidectomized (Tx) rats was significantly ($P<0.05$) increased compared to euthyroid animals. Five days after Tx, there was a three-fold increment in both organs and enzyme activity remained elevated 2 weeks later.

Fractional turnover rate (FTr) and biological half-life ($t_{1/2}$)

One hour after cycloheximide administration, AG-5'D activity decreased 57% and maximal inhibition (87%) was attained 3 h postinjection (Figure 8). Linear regression analysis of these data (inset) allowed calculation of the fractional disappearance rate (0.68 h^{-1}), half-life (1.01 h) and the production rate (108 U/mg/h).

Discussion

Present results demonstrate that in both functional components of the rat AG (cortex and medulla), the major iodothyronine deiodinase activity corresponds to the isotype 5'D-II. Adrenal gland deiodinase activity exhibits a Km for T4 and rT3 in the nmolar range, the affinity for the former being nine times higher. The enzyme is resistant to PTU and GTG; and its reaction kinetics show a typically 'sequential' pattern. Furthermore, in accordance with *in vivo* and *in vitro* studies (Kaplan, 1980, Leonard *et al.*, 1981, 1990), present

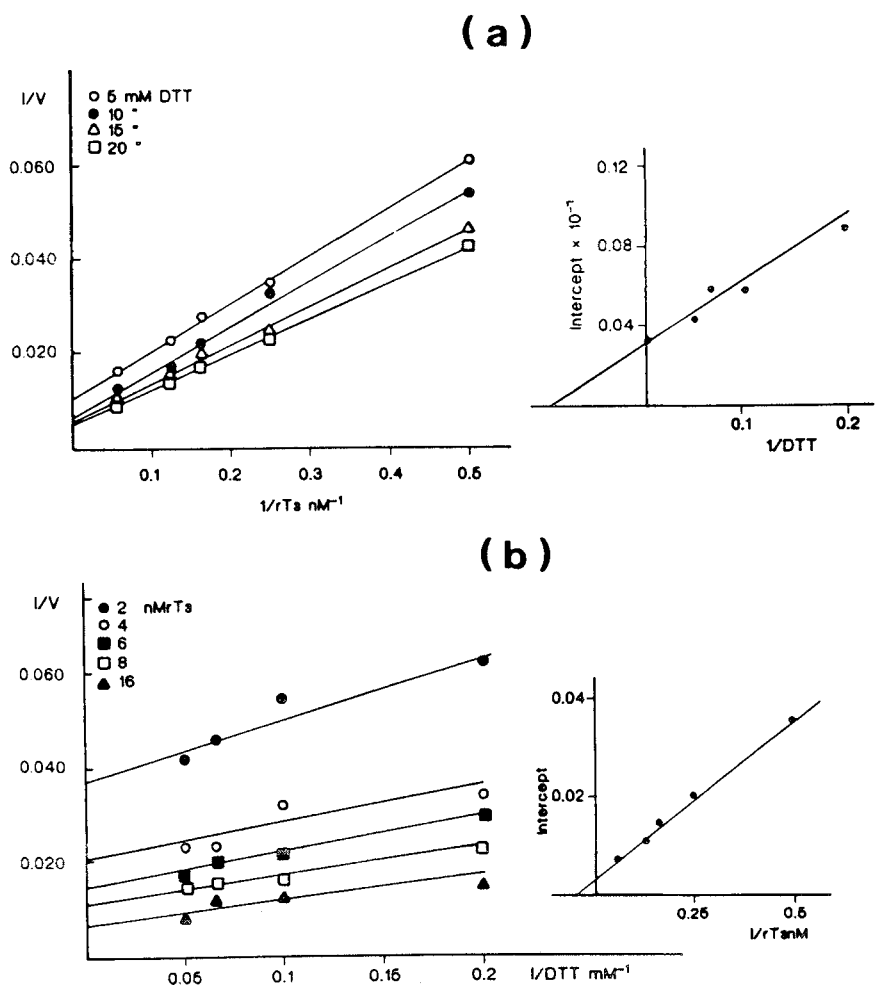

Figure 4 Kinetics of AG-5'D as a function of DTT. (**a**) Double-reciprocal plot of the rate of rT_3 5'-deiodination by 150 μg of Mc fraction as a function of substrate rT_3 concentration in the presence of -○-, 5; -●-, 10; -Δ-, 15 and -□- 20 mM DTT. Each point represents the mean ± S.E. from 3 independent experiments in duplicate. *Inset*: Replot of intercepts as a function of reciprocal DTT concentration, linear regression allowed to calculate the true Km and Vmax for rT_3. (**b**) The Lineweaver-Burk plots of deiodination rate *vs* DTT concentration in presence of ● 2, ○ 4, □-6, ■ 8 and ▲ 16 nM rT3. *Inset*: Replot of intercepts as a function of reciprocal rT3 concentration. The corresponding true Michaelis-Menten constants are summarized in Table 1

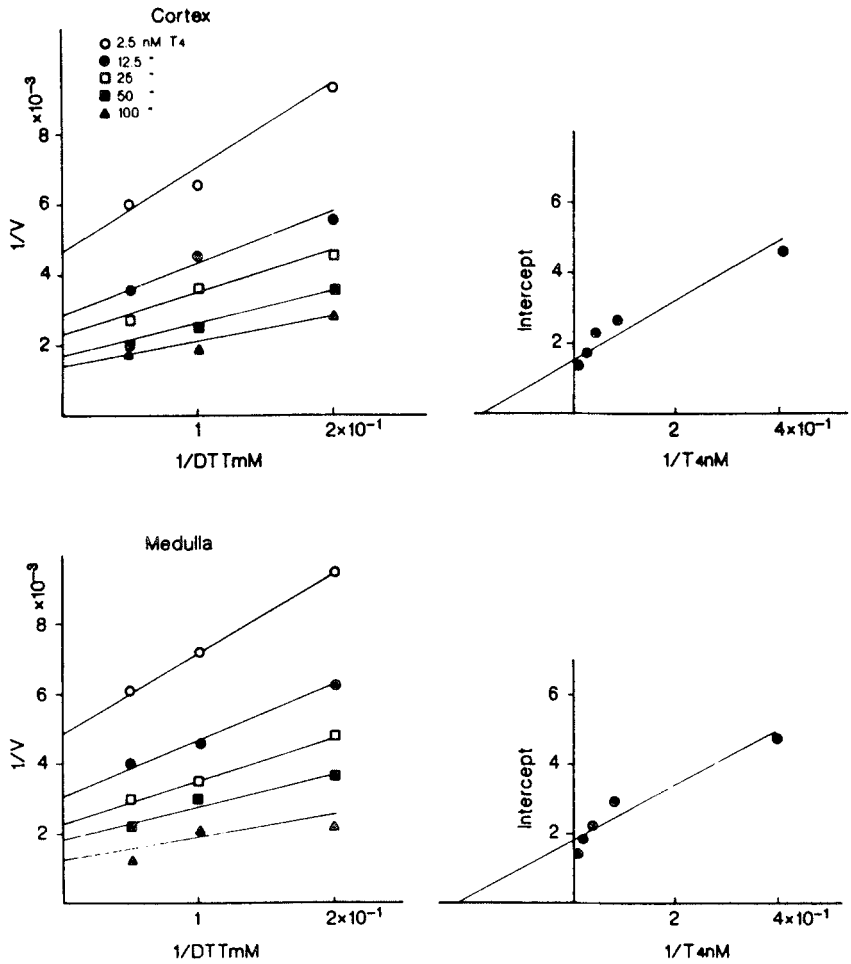

Figure 5 Kinetic analysis of T4-5'D in adrenal cortex and medulla. 5'D-11 was determined at concentrations of ○, 2.5; ●, 12.5; □, 25; ■, 50; and ▲ 100 nM T4, 1 mM PTU and 5, 10 and 20 mM DTT. Each point represents the mean ± S.E. of one experiment by triplicate. (a) Double reciprocal plot, of 5'D-11 activity in adrenal cortex as a function of DTT. *Inset*: Replot of intercepts as a function of reciprocal T4 concentration; linear regression allowed to calculate the true Km and Vmax for T4. (b) The Lineweaver-Burk plots of adrenal medulla 5'D-11 activity *vs* DTT concentration in presence of T4. *Inset*: Replot of intercepts as a function of reciprocal concentration of DTT The corresponding true Michaelis-Menten constants are summarized in Table 1

results in Tx-rats demonstrate that AG-5'D-II activity is inversely related to thyroid hormone supply. This finding agrees with our previous reports in which AG-5'D activity increased (two-fold) after hypophysectomy (Anguiano *et al.*, 1991); as well as with recent data showing that when these animals received replacement treatment with T4 (1 μg/100 g W, i.p.) enzyme activity returned to control values (Anguiano *et al.*, 1993).

On the other hand, the present finding on the short half-life and rapid turnover rate of the adrenal gland enzyme is a further peculiarity which characterizes this enzyme in other tissues such as pituitary, CC and BAT (Kaplan, 1980; Visser *et al.*, 1982; Leonard *et al.*, 1984; Guerrero & Reiter, 1992). In a closely related study (Luna *et al.*, 1994 submitted), we report that AG 5'D-II activity exhibits a nyctohemeral rhythm with acrophase at approximately 2:30 h. This finding, as well as the short half-life and rapid turnover rate of 5'D-II activity, seems to be a conspicuous functional distinction among the two isoenzymes that catalyze outer-ring thyronine deiodination. Furthermore, these data strongly suggest that beside the plausible increase in its $t_{\frac{1}{2}}$, 5'D-II synthesis may also be increased during the dark period. Previous studies have reported that in hypothyroid animals, the half-life of 5'D-II is significantly increased with the consequent fall in FTr both in adenohypophisis and in cultured glial cells (Leonard *et al.*, 1984, 1990). These reports have related thyroid hormone effects with the modulation of enzyme inactivation and degradation (Leonard *et al.*, 1990).

Sensibility of 5'D-II to PTU in medulla and cortex denote no significant differences in the proportion of isoenzyme contents of these structures. However, we cannot discard the hypothesis that enzymatic activity may be regulated by different factors since, as is well known, the cortex and medulla are of different embryological origin. We propose that the immediate activation induced by acute exposure to cold is partially originated in the medulla and that it is activated by a posttranslational transformation.

Materials and methods

Reagents

Nonradioactive iodothyronines were obtained from Henning Co (Berlin, Germany), and PTU and GTG from Sigma Chemical Co. (St Louis, MO). ^{125}I-labeled T_4 and rT_3 (sp.a. 1200 and 1174 μCi/μg, respectively) were purchased from New England Nuclear (Boston, MA). Dithiothreitol (DTT) and cycloheximide were obtained from Calbiochem (La Joya, CA).

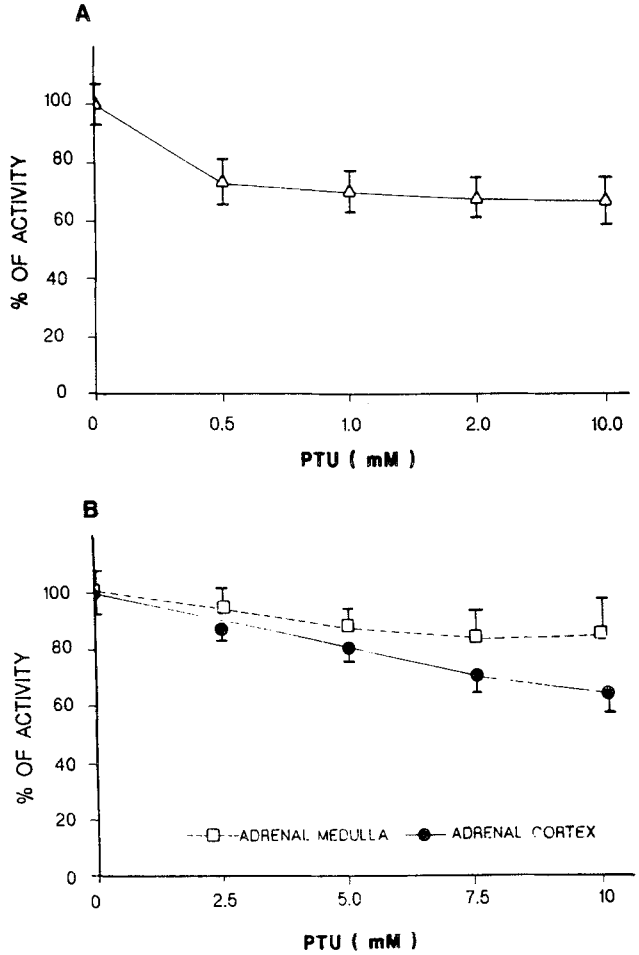

Figure 6 Effect of PTU in AG-5'D. Enzyme activity was assayed in the presence or absence (control) of different concentrations of PTU. Results are expressed as the percentage of change, compared to controls which represent 100% of the activity. Bars indicate S.E. of three independent experiment in duplicate (**A**) *Whole adrenal gland.* With 10 mM PTU total enzyme activity was inhibited ≈ 30%. (**B**) *PTU sensitivity in medulla and cortex.* These experiments were carried out using Mc fractions. PTU inhibited only 15% of medulla 5'DII activity, whereas in the cortex the enzyme inhibition was 30%

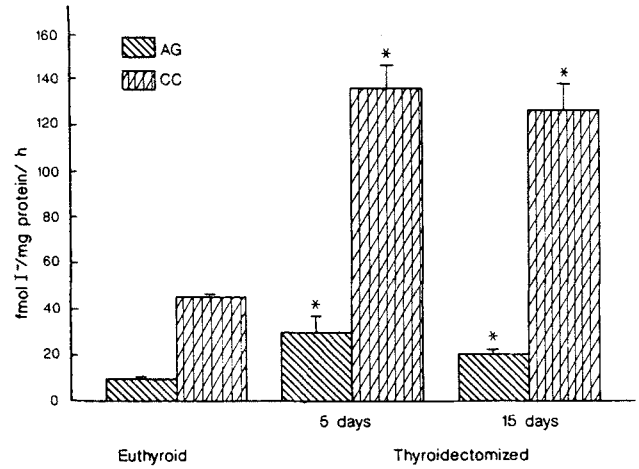

Figure 7 Effect of thyroidectomy on AG5'D-II. Time course of changes in AG and cerebral cortex (CC) 5'D-II deiodination after thyroidectomy. The Mc fractions were prepared from euthyroid (control) and surgically thyroidectomized rats (Tx) for 5 and 15 days. The assay condition for CC and AG were similar for both as described in *Materials and methods.* Each data point represent the mean ± S.E. of specific activity from five animals. Statistical analysis, included Bonferroni's test and one way-analysis of variance. Asterisk indicates $P<0.05$

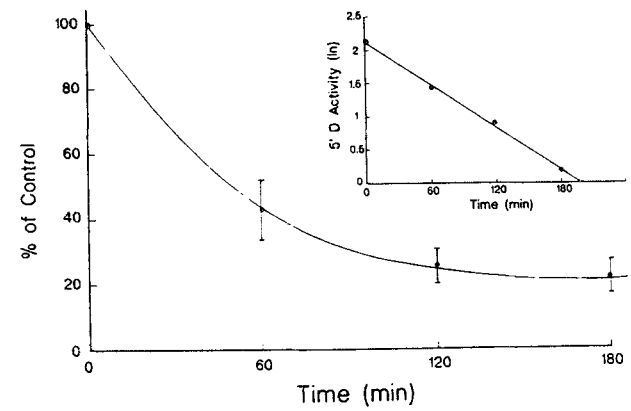

Figure 8 5'D-II fractional turnover rate. Cycloheximide (6 mg/100 g wt) was injected i.p. at time 0. Each point represents the mean value of 5–6 rats ± S.E. Basal level of 5'D activity was 10 fmol I/mg protein/hr, which represents 100% of the activity. *Inset*: semilog plot of the same results

Animal housing and sample collection

Euthyroid or surgically thyroidectomized (Tx) male Wistar rats weighing 250–300 g were kept under a 12:00/12:00 light:dark cycle in a temperature controlled room (22 ± 1°C), and were provided with water and standard rat pellets (Purina Lab Chow) *ad libitum.* Procedures regarding care, treatment administration and euthanasia of animals were reviewed and approved by an *ad hoc* ethic committee. All animals were decapitated at 09:00 h and the cerebral cortex and adrenal glands were immediately removed. Adrenal glands (AG) were dissected in their two functional compartments (cortex and medulla) with the aid of a stereoscopic microscope. These tissues were homogenized in 1:10 (w/v) ice-cold 10 mM HEPES buffer (pH 7.5), containing 0.25 M sucrose and 1 mM EDTA. Crude homogenates were centrifuged at 10 000 *g*, for 15 min at 4°C. When not assayed immediately, the supernatant of 10 000 *g*, hereafter referred to as microsomal crude fraction (Mc fraction), was quick-frozen in dry ice-acetone and stored at − 70°C until assayed. Protein content was measured by Bradford's method and hypothyroidism was confirmed by measurement of serum T_4 and T_3 (Valverde-R C. *et al.*, 1989).

Deiodination assay

5'D activity was measured using either T_4 and quantifying the generated T3 by RIA (Valverde-R & Aceves, 1989), or by the radiolabeled iodide release method described elsewhere (Anguiano *et al.*, 1991; Luna *et al.*, 1993). For the T_3 production assay, 150–250 µg of Mc-fraction of whole gland and separated cortex or medulla were incubated with unlabeled T_4 (2.5–100 nM); 5–20 mM DTT; with or without PTU for 3 hr at 37°C. The T_3 generated during incubation was quantified by RIA of an ethanol extract of the incubation mixture. Results are expressed in specific activity as femtomoles of T_3 produced/mg protein/h and represent the mean ± S.E.

In the case of the rT_3 deiodination assay the reaction mixture (115 µl) consisted of rT_3 (2–400 nM rT_3 containing 4 nM of ^{125}I-rT_3); DTT, 5–20 mM and PTU. The reaction was stopped by adding 50 µl of a cold solution containing 50%

normal bovine serum plus PTU 10 mM, and 350 μl of 10% trichloroacetic acid. After centrifugation (3000 r.p.m. × 10 min) the supernatant was decanted onto a column (Dowex-50X) and eluted with 2 ml of 10% acetic acid. The ^{125}I eluate, which is an index of iodothyronine 5'D activity, was determined in a gamma-spectrometer. Data are expressed in specific activity as femtomoles I^- released/mg of protein/h. A unit of 5'D is defined as 1 fmol of rT_3 deiodinated/h (U).

Fractional turnover rate (FTr)

Groups of five animals each, received a single i.p. dosis of 100 μl cycloheximide (6 mg/100 g wt was dissolved in 100 μl ethanol-H_2O 1:9, vol/vol). Control animals received 100 μl of vehicle. Animals were killed by decapitation 60, 120 and 180 min postinjection. The FTr of 5'D-II was determined from linear regression analysis of semilog plots of the enzyme activity at each time point after the injection of cycloheximide. The enzyme production rate (units per mg protein/h) was calculated as the product of the FTr multiplied by the steady-state enzyme levels (units per mg protein) (Leonard *et al.*, 1984, 1988).

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