

# Comparison of Iodothyronine 5'-Deiodinase and Other Thyroid-Hormone-dependent Enzyme Activities in the Cerebral Cortex of Hypothyroid Neonatal Rat

## EVIDENCE FOR ADAPTATION TO HYPOTHYROIDISM

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**ABSTRACT** Recent studies have shown that ~75% of the nuclear 3,5,3'-triiodothyronine ( $T_3$ ) present in adult rat cerebral cortex (Cx) derives from 5'-deiodination of thyroxine ( $T_4$ ) within this tissue. The activity of iodothyronine 5'-deiodinase (I 5'D), the enzyme catalyzing  $T_4$  to  $T_3$  conversion, increases rapidly after thyroidectomy, suggesting that this could be a compensatory response to hypothyroxinemia. To evaluate this possibility during the period of central nervous system maturation, we studied several thyroid hormone-responsive enzymes (aspartic transaminase [AT], succinic dehydrogenase [S.D.], and Na/K ATPase) in the Cx of 2-, 3-, and 4-wk-old rats. The rats were made congenitally hypothyroid by placing 1, 2, 5, and 20 mg methimazole (MMI) in 100 ml of the mothers' drinking water from day 16 of gestation throughout the nursing period and to the litters after weaning. In addition, serum thyroid hormones, I 5'D, and, in some experiments, *in vivo*  $T_4$  to  $T_3$  conversion in Cx were measured in the same pups. Serum  $T_4$  concentrations varied from <1 to 40 ng/ml and were generally inversely related to maternal MMI dose. Serum  $T_3$  was less affected by MMI than was  $T_4$ . At 2 wk, decreases in AT, S.D., and ATPase were present in the 20-mg-MMI group but not in the 5-mg-MMI pups despite low serum  $T_4$  (<10 ng/ml) in the latter. At 3 and 4 wk, both 5- and 20-mg-MMI groups had significant reductions in these cortical enzymes despite a normal serum  $T_3$  in the 5-mg-MMI rats. Cortical I 5'D activity was 10-fold the control value in 5- and 20-mg-MMI

animals at 2 wk but increased only three- to fivefold at 3 and 4 wk. I 5'D correlated inversely with serum  $T_4$  ( $r \geq 0.96$ ) at all ages, but the less marked elevation of this enzyme in 3- and 4-wk-old pups was not accompanied by an increase in serum  $T_4$ . Serum  $T_3$  increased or remained the same between 2 and 3 wk. These results suggested that the 10-fold increase in I 5'D at 2 wk protected the 5-mg-MMI group from tissue hypothyroidism, but that the three- to fivefold increase at 3 and 4 wk could not. Injection of ~250 ng  $T_4$ /100 g body weight to 2-wk-old, 20-mg-MMI pups (one-sixth the normal  $T_4$  production rate) led to both a 1.8-ng/g cortical tissue increment in cortical  $T_3$  and a significant increase in AT at 24 h, compared with a 0.38-ng/g cortical tissue  $T_3$  increment and no change in AT in euthyroid controls. The larger increment in  $T_3$  of the 20-mg-MMI pups was due in great part to increased fractional  $T_4$  to  $T_3$  conversion. Although the latter resulted in greater serum  $T_3$  concentrations, three-fourths of the newly formed  $T_3$  in the cortex was generated *in situ*, and it was blocked by iopanoic acid as was the increase in AT. We conclude that 70–80% of the  $T_3$  in the Cx of the neonatal rat is produced locally. Serum  $T_4$  appears to serve both as a precursor for  $T_3$  and as a critical signal for increases in cortical I 5'D. The increased I 5'D can result in normal or near-normal cerebrocortical  $T_3$  concentrations despite marked reductions in serum  $T_4$ . This mechanism seems to be particularly effective around 2 wk of age when many thyroid-hormone-dependent maturational changes occur in the rat Cx.

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## INTRODUCTION

The concept that thyroid hormones are essential for normal development of the central nervous system

(CNS)<sup>1</sup> is well established (1). The lack of these hormones during critical periods of CNS maturation induces morphological, physiological, and biochemical abnormalities. The most striking morphological alteration is a reduction in the number of arborizations of the neurons in the cortex of cerebral hemispheres and cerebellum (2). These perturbations induced by thyroid hormone deficiency are paralleled biochemically by a decrease in the incorporation of glucose-carbon into glutamic acid in the so-called gamma aminobutyric acid shunt (3). Both aspartic transaminase (AT) and succinic dehydrogenase (S.D.), enzymes involved in these metabolic pathways, are known to be decreased in the brain of neonatal hypothyroid rats (4, 5), although a number of other enzymes not as closely related to this pathway have also been shown to be reduced (5).

We have recently found that *in situ* thyroxine (T<sub>4</sub>) to triiodothyronine (T<sub>3</sub>) conversion is the main source of T<sub>3</sub> in adult rat brain, accounting for 75 and 60% of the T<sub>3</sub> measurable in cerebral cortex (Cx) and cerebellar cell nuclei, respectively (6). Enzymatic 5'-deiodination of T<sub>4</sub> is demonstrable in homogenates of different regions of the brain (7). Even more interesting is that this enzyme activity has a maturational pattern that suggests that it may be of physiological relevance for the development of the brain (8), because the activity is increased between 10 and 30 d when most of the changes alluded to above take place (1-5). The finding of increased enzyme activity in adult (7, 9, 10), as well as in neonatal, hypothyroid rat cerebral homogenates (11) further supports that suggestion.

Earlier studies from our laboratory showed that the pituitary T<sub>3</sub> content is also heavily dependent on local T<sub>4</sub> to T<sub>3</sub> conversion (12). A fall in T<sub>4</sub> production resulting from a failing thyroid gland leads to pituitary thyrotropin (thyroid-stimulating hormone [TSH]) release, and the ensuing thyroid stimulation maintains plasma T<sub>3</sub> within normal limits until well advanced thyroid failure (for a review see 13). Studies of congenitally hypothyroid infants show that the level of serum T<sub>3</sub> is often normal, whereas that of T<sub>4</sub> and TSH are markedly altered (14).

It thus seems possible that the ultimate impact that thyroid hormone deficiency may have on brain development depends not only on the extent of the thy-

roid failure, but also on the success of the various compensatory mechanisms in the pituitary and in the brain itself. Because at birth both the CNS and hypothalamic-pituitary system of the rat are less mature than those in the human newborn (15, 16), the rat may be a suitable model for evaluating the impact that different degrees of thyroid failure may have on human brain development *in utero*. Accordingly, we have induced congenital hypothyroidism in rats by giving pregnant dams various concentrations of methimazole (MMI) in the drinking water from day 16 of gestational age (GA), i.e., before the thyroid of the fetus is functioning (15), throughout the nursing period. Because the most critical changes in brain development occur between 10 and 21 d postnatally (2, 3), the responses to hypothyroidism at 2 wk were compared with those at later ages.

## METHODS

### *Rats and experimental protocols*

Pregnant Sprague-Dawley rats were obtained from Zivic-Miller Laboratories (Allison Park, PA) on day 15 or 16 of gestation. They were housed in individual cages, and on day 16 of GA they were given tap water ad lib. with 0, 1, 2, 5, and 20 mg MMI/100 ml water (1-, 2-, 5-, and 20-mg-MMI groups). This regimen was maintained throughout the nursing period (up to 4 wk), at which time control rats and some of the litters receiving the lowest concentration of MMI had weaned, but were kept in the same cage with MMI-containing water. Within the first 24-48 h after birth, litters were made nine to eleven rats each by redistribution of the pups within dose groups; subsequently the number of rats per litter was kept as nearly equal as possible. Because several litters with the same treatment were run simultaneously, it was always possible to redistribute the pups and make up the groups from two to three litters for each experiment. However, due to a significant mortality rate (three to five rats/litter in 4 wk), and in order to examine the same litters through the 4-wk period, experimental groups were limited to four to five rats. Unless otherwise stated, the variables analyzed in a given experiment were all measured in the same pups. Within these MMI concentration ranges the dams did not restrict their food and water intake (40-70 ml/d). A number of pilot experiments were performed to determine whether the maternal thyroid status influenced either the nutritional condition or the thyroid status of the offspring. Mothers treated with the highest concentration of MMI received T<sub>3</sub> simultaneously (0.2 µg/ml) in drinking water containing 0.01% bovine serum albumin (BSA). At age 3 wk these pups weighed 29±4 g and their serum T<sub>3</sub> was 0.09±0.03 ng/ml as compared with 29±4 g and 0.16±0.08 ng/ml in the pups of mothers not receiving the hormone (n = 5). The mothers' serum T<sub>3</sub> concentrations were 0.40 and <0.03 ng/ml, respectively. In other experiments, the 20-mg-MMI-treated dams were implanted with an osmotic minipump (Alza Corp., Palo Alto, CA) to deliver ~500 ng T<sub>3</sub>/100 g body weight (bw)/d; the minipump was replaced at 14 d as indicated by the manufacturer. At 3 wk, T<sub>3</sub>-treated mothers had a serum T<sub>3</sub> of 2.5 ng/ml as compared with <0.03 in the T<sub>3</sub>-untreated dams. The pups weighed 33±1 and 35±2 g and their serum T<sub>3</sub> concentrations were 0.16±0.06 and 0.17±0.08

<sup>1</sup> *Abbreviations used in this paper:* AT, aspartic transaminase; bw, body weight; CNS, central nervous system; Cx, cerebral cortex; DTT, dithiothreitol; GA, gestational age; α-GPD, α-glycerophosphate dehydrogenase; INT, iodonitrotetrazolium; IOP, iopanoic acid; I 5'D, iodothyronine 5'-deiodinase; MMI, methimazole; PTU, propylthiouracil; rT<sub>3</sub>, reverse T<sub>3</sub>; S.D., succinic dehydrogenase; TSH, thyrotropin (thyroid-stimulating hormone); T<sub>4</sub>, thyroxine; T<sub>3</sub>, triiodothyronine.

ng/ml, respectively ( $n = 5$ ). Finally, the stomachs of the pups, usually full of milk, were removed at death and found to weigh  $1.03 \pm 0.41$  g ( $n = 7$ ) and  $1.15 \pm 0.7$  g ( $n = 8$ ) in controls and MMI-treated litters, respectively, at age 2 wk. These results indicated there was no significant effect of maternal thyroid status on the pups.

The pups were weighed only on the day of the experiment. They were killed either by decapitation or by aortic exsanguination if radioisotopes had been injected (see below). Cx was removed *in situ* with the aid of a fine spatula; it was usually contaminated with some white matter but basal ganglia were excluded. Between 600 mg (2 wk) and 900 mg (4 wk) of tissue was collected. Except occasionally at age 2 wk, there was no difference between the amount of tissue collected from control and from MMI-treated rats. The liver was removed almost *in toto* yielding between 0.7 and 1.5 g of tissue, depending on the age and on the thyroid status of the pups. Both tissues were immediately homogenized in four volumes of 0.32 M sucrose, 10 mM Hepes pH 7.0, and 1 mM dithiothreitol (DTT) (liver) or 10 mM DTT (cortex) (17). They were then either snap frozen in dry ice-acetone or kept in ice-cold water for assay within 1–2 h of death, as indicated below. All assays of a given age group were performed simultaneously.

### Enzyme assays

**Succinic dehydrogenase.** Assay was performed in crude homogenate based on the method of Slater and Bonner (18) with some modifications. The total reaction volume was 3 ml with this final composition: 0.03 M Na succinate; 0.01 M KCN; 0.001 M  $K_3Fe(CN)_6$ ; and 0.12 M potassium phosphate buffer, pH 7.2, containing 0.5% BSA. The tissue sample was added in 0.2-ml aliquots representing ~2–3 mg of protein or 40–60  $\mu$ g of DNA. After the tubes were preincubated for 5 min at 37°C, prewarmed  $K_3Fe(CN)_6$  was added quickly and the reaction started by the addition of Na succinate. The reaction was stopped by adding 2 ml of 10% ice-cold TCA at 1, 2, and 4 min. Blanks were made by adding the TCA before the  $K_3Fe(CN)_6$  and Na succinate. The tubes were centrifuged at 2,000 g for 10 min and the optical density at 400 nm determined. Under these conditions, the reaction was linear with time and with homogenate concentration up to 5 mg protein/assay tube. Results are expressed in  $\Delta$  optical density units per minute per milligram DNA (see below). In later experiments, S.D. was assessed using iodinitrotetrazolium (INT) as electron acceptor (19), which turned out to be simpler and faster. Total reaction volume was 0.27 ml: 100  $\mu$ l of 0.12 M  $KPO_4$ , 0.5% BSA, pH 7.2; 50  $\mu$ l of sample as a 1:10 dilution of crude homogenate in buffer (50–75  $\mu$ g protein); 20  $\mu$ l of 0.1 M KCN in water; and 50  $\mu$ l of 4 mg/ml INT in water. After a preincubation of 5 min at 37°C the reaction was started by the addition of 50  $\mu$ l of 0.15 M Na succinate, pH 7.2, in water. Only water was added to the blanks. The reaction was stopped at 15 min by adding 250  $\mu$ l of 10% ice-cold TCA, followed by 2 ml of 95% ethanol. Tubes were centrifuged at ~2,000 g for 10 min and the optical density determined at 500 nm. The reaction was linear with time up to 20 min, provided the optical density was <0.600. The optical density of the blank tubes varied between 0.02 and 0.05, and the samples between 0.15 and 0.35. As to the effect of MMI-treatment, both methods gave the same results, i.e., the 20-mg-MMI pups showed 30–50% lower values than the controls (see Results).

**Aspartic transaminase.** The assay was performed using crude homogenates; the method used was based on that de-

signed by Karmen (20), but slightly modified. The homogenate was pretreated with deoxycholate to release the latent enzyme (21). Usually 0.4 ml of one-fifth homogenate was incubated for 20 min at 37°C with 0.15 ml of 4.25% Na deoxycholate, and it was subsequently diluted 1:40 with the reaction buffer (0.01 M sodium phosphate, pH 7.4). The reaction mixture contained 4.2 ml of buffer; 0.6 ml of 0.2 M Na  $\alpha$ -ketoglutarate, pH 7.4; 0.3 ml 500 U/ml malic dehydrogenase (in phosphate buffer); and 1.2 ml diluted homogenate. Sets of four tubes were immersed in a water bath at 30°C and preincubated for 5 min, and then 0.6 ml of 1 mg/ml NADH solution was added, rapidly followed by 1.5 ml of 0.2 M Na aspartate, pH 7.4, at 15-s intervals. Each tube was read at 340 nm for 3–4 min at 1-min intervals in a Gilson spectrophotometer (Gilson Medical Electronics, Inc., Middleton, WI) with an aspirating rapid sampler. Reaction velocity was linear with time (up to 5 min with euthyroid cortex homogenate and even longer in hypothyroid rat homogenates) and with protein concentration. Results are expressed as  $\Delta$  optical density per minute per milligram DNA (see below).

**Na/K-dependent ATPase.** This was measured by the release of P from ATP in the presence and absence of 1 mM ouabain (22). Total assay volume was 0.25 ml and the reaction mixture contained 0.100 M Hepes, pH 7.4, 0.1 M NaCl, 0.02 M KCl, 6 mM ATP with 6 mM  $MgCl_2$ , and  $\pm$ 1 mM ouabain. 25 and 50  $\mu$ l of crude homogenate diluted 1:15 in water (20–60  $\mu$ g protein) were usually assayed. Incubation was for 30 min at 37°C. Results are expressed as micromoles of P released per minute per milligram DNA (see below).

**Iodothyronine 5'-deiodination (I 5'D) activity.** This was assayed by two methods in crude homogenates of cortex and liver: by  $T_4$  to  $T_3$  conversion and by 5'- $^{125}I$ -reverse  $T_3$  ( $rT_3$ ) deiodination. Assay conditions, procedure, and product separation were recently described in detail (17). The  $T_4$  concentration was 2 nM (cortex) or 100 nM (liver), along with ~100,000 cpm/tube of [ $^{125}I$ ] $T_4$  (sp act ~4,200  $\mu$ Ci/ $\mu$ g).  $rT_3$  concentration was also 2 nM. Assays were performed in the presence of 20 mM (cortex) or 1 mM (liver) DTT. 1 mM propylthiouracil (PTU) was added to the  $rT_3$  deiodination assay because, in the cortex, the PTU-insensitive  $rT_3$  5'-deiodination, at 2 nM  $rT_3$ , is indistinguishable from  $T_4$  to  $T_3$  conversion at 2 nM  $T_4$  concentration (17, 23, 24). Results were expressed as either moles of  $T_3$  formed or  $rT_3$  deiodinated per hour per milligram DNA (cortex) or of protein (liver).

The Cx enzyme activities have been expressed on a DNA basis rather than on the more conventionally used protein basis. The rationale for this is that the Cx protein:DNA ratio is decreased in hypothyroid animals but it increases with age and body weight. Thus, protein:DNA ratio (milligram per milligram) in cortex homogenates of 2-wk-old 20-mg-MMI pups ranged from 30 to 70 for body weights of 18 to 30 g; the corresponding figures for controls were 40 to 90 for body weights between 38 and 48 g. On the other hand, the DNA content is fairly constant during the neonatal period in both controls and hypothyroid pups (4, 5). This method of expressing the results has been shown to be more sensitive (4, 5) and allows a better assessment of the enzyme on a per cell basis.

**Hepatic mitochondrial  $\alpha$ -glycerophosphate dehydrogenase ( $\alpha$ -GPD).** Liver homogenate was made 1:10 with 0.32 M sucrose and 1 mM  $MgCl_2$ , and then centrifuged at 800 g for 10 min. The supernatant was centrifuged at 10,000 g for 10 min and the pellet was washed once in 0.32 M sucrose and 1 mM  $MgCl_2$ . The pellet was resuspended in 2 ml of 0.05 M phosphate buffer, pH 7.4. The assay was performed

as modified by Gardner (25) and Okamura et al. (26) but using 0.05 M phosphate buffer instead of 3-(*N*-morpholino) propanesulfonic acid buffer as the latter authors did. The omission of phenazine methosulfate proposed in the original method by Lee and Lardy (27) reduces the amount of the colored product iodonitroformazan by 50–60% (25). Thus the optical density at 500 nm is reduced but the activity ratio of euthyroid to thyroidectomized is the same, i.e., ~10.

None of these enzyme activities was affected by adding 1 mM MMI *in vitro*, nor by preincubating the homogenate with that concentration of the drug. The highest concentration of MMI in the drinking water was 1.75 mM (20 mg/dl). Pilot experiments in adult thyroidectomized rats with undetectable serum T<sub>3</sub> and T<sub>4</sub> concentrations showed that MMI in the drinking water at 20 mg/100 ml for 2 mo did not affect the level of any of these enzymes. This indicates that the observed effects of MMI in the newborn rats are due to hypothyroidism and not nonspecific effects of the MMI.

### *In vivo studies*

To assess the capacity of both the cortex and liver to generate T<sub>3</sub> from T<sub>4</sub> *in vivo*, small doses of radiolabeled T<sub>4</sub> were injected intravenously. In the first of these experiments <sup>131</sup>I-T<sub>4</sub>, 600 ng/100 g bw (sp act ~5800 μCi/μg), was injected and animals were killed 16 h later. In the second experiment <sup>125</sup>I-T<sub>4</sub>, 60 ng/rat (150–300 ng/100 g bw), was injected 24 h before death followed in 8 h by the injection of tracer [<sup>131</sup>I]T<sub>3</sub> subcutaneously. The latter was used to estimate what fraction of the observed [<sup>125</sup>I]T<sub>3</sub> in the tissues was accounted for by the plasma [<sup>125</sup>I]T<sub>3</sub> (6). In these experiments, iopanoic acid (IOP) was given to some rats intraperitoneally before the injection of labeled T<sub>4</sub> (see Results). [<sup>125</sup>I]T<sub>3</sub> and [<sup>131</sup>I]T<sub>3</sub> in the serum were measured by a combined affinity and paper chromatography method described previously (28). Serum [<sup>125</sup>I]T<sub>4</sub> was measured by descending paper chromatography of whole serum (29) or by TCA precipitation. Iodothyronines were extracted from crude homogenate in butanol saturated with 2 N HCl, usually 0.25 ml homogenate plus 1 ml of butanol HCl, which ensured >80% extraction. The extract was dried under N<sub>2</sub> and the iodothyronines resuspended in 50 μl ethanol: 2 N NH<sub>4</sub>OH (99:1) containing 1 mg/ml T<sub>4</sub>, T<sub>3</sub>, and NaI. The suspension was applied to 3-mm Whatman paper (Whatman Inc., Clifton, NJ) and developed as described (29).

### *Other procedures*

T<sub>4</sub>, T<sub>3</sub>, and TSH were assayed in serum by methods previously reported (30–32). T<sub>4</sub> and T<sub>3</sub> from hypothyroid rats were measured using hormone-stripped serum from hypothyroid rats in the standard curves. Hypothyroid rat serum decreases the slope of the displacement curve in such a way that samples with low T<sub>3</sub> values are overestimated if read using standard curves containing hormone-free serum from euthyroid rats.

Results are expressed as mean±SD or range. Statistical analyses included *t* tests, both paired and unpaired, and regression analyses. The latter were performed using a Hewlett-Packard 9815A calculator (Hewlett-Packard Co., Palo Alto, CA) by evaluating the best fit of the data to a number of linear and nonlinear mathematical models contained in the software from the manufacturer. This was done merely to facilitate the description of the data. The interpretation

of the better fit to one model as compared with another is beyond the scope of this work. Similarly, we intend no implication as to whether the relationships implied by the formulas would predict the changes in *y* beyond the observed ranges of the variable *x*. As to the comparisons between the mean of a given variable obtained with a given dose of MMI and that of the control group within an experiment, i.e., at one particular age (Table I, Fig. 1), we used a per comparison approach (33) rather than a multiple comparison test (34). The rationale for this statistical treatment is that we do not attach any significance to each dose of MMI, which was arbitrarily chosen to induce graded hypothyroidism, but to the serum thyroid hormone concentrations induced by this treatment and their relationship to the biological responses analyzed. Because serum levels of T<sub>3</sub> and T<sub>4</sub> varied widely both within and between treatment groups, we further evaluated the data in terms of the individual hormone results, regardless of the MMI dose that induced them.

## RESULTS

*Effect of MMI given to the mothers on serum hormone levels of the offspring (Tables I and II and Fig. 1).* Serum T<sub>4</sub> in control pups did not change with age, but serum T<sub>3</sub> increased between 2 and 3 wk to a plateau as has been described (15) (Table I). Such a pattern was observed also in the 1-mg-MMI group (*P* < 0.01), but was not evident in the pups exposed to higher concentrations of MMI. The various concentrations of MMI produced a wide spectrum of serum T<sub>4</sub> and T<sub>3</sub> concentrations even within a given group, probably reflecting individual variations in the MMI intake through the milk, the susceptibility of the thyroid to the drug, and the response of the pituitary thyroid axis, etc. (compare the standard deviations of MMI-treated pups with those of the control groups). The 1-mg-MMI group serum T<sub>3</sub> was not different from controls at any age, but the 5-mg-MMI group serum T<sub>3</sub> became significantly lower at 4 wk. At all ages, the 20-mg-MMI group had significantly lower serum T<sub>3</sub> concentrations than the controls. Serum T<sub>4</sub> was always decreased by MMI except in the 1-mg-MMI group at 2 wk, where there was an overlap with the controls. Fig. 1 shows the relationship between the individual serum concentrations of both iodothyronines and gives a better idea of the variation in the response to MMI. The data were best fit to a model where  $[T_3] = a [T_4]^b$ . At all ages, but especially at 2 and 3 wk, serum T<sub>3</sub> fell below the normal range only when T<sub>4</sub> was very low. At 3 wk and, to some extent, at 4 wk, supranormal levels of T<sub>3</sub> (>0.65 ng/ml) were often found with normal or low serum T<sub>4</sub> values.

Because of the short supply of serum, TSH was measured in a limited number of samples. Table II shows the results in samples from 2½- to 3-wk-old pups. The range of response was broad, as is evident from the standard deviation within each MMI-dose group. The 5-mg-MMI group showed the highest levels of TSH

TABLE I  
Serum T<sub>4</sub> and T<sub>3</sub> Levels in Neonatal Rats with MMI-induced Congenital Hypothyroidism

Age	Control		1		2		5		20	
	T <sub>4</sub>	T <sub>3</sub>	T <sub>4</sub>	T <sub>3</sub>	T <sub>4</sub>	T <sub>3</sub>	T <sub>4</sub>	T <sub>3</sub>	T <sub>4</sub>	T <sub>3</sub>
wk										
Exp. 1										
2	36±3.8	0.41±0.10	27±8.0 <0.025‡	0.37±0.08 NS‡	—	—	2.6±3.2 <0.001	0.30±0.10 NS	0.9±0.7 <0.001	0.19±0.05 <0.010
3	33±4.0	0.62±0.05§	29±2.6 NS	0.78±0.20§ NS	—	—	13.0±9.4 <0.01	0.58±0.25 NS	2.3±3.2 <0.001	0.36±0.18 <0.05
4	37±2.2	0.65±0.03 <sup>  </sup>	21±4.3 <0.001	0.67±0.12§ NS	—	—	2.6±0.6 <0.001	0.32±0.04 <0.001	4.1±6.0 <0.001	0.25±0.05 <0.001
Exp. 2										
2½	34±3.3	0.47±0.04	—	—	21±7.7 <0.025	0.69±0.20 <0.005	16±7.3 <0.005	0.41±0.18 NS	1.1±0.8 <0.001	0.16±0.04 <0.001

Data represent mean±SD.

\* MMI was given to pregnant rats in drinking water from day 16 of gestation throughout the nursing period as described in Methods. The drug concentration is given as mg/100 ml. Each set of T<sub>4</sub> and T<sub>3</sub> values represents the mean±SD of four pups.

‡ P value when compared with age-matched controls.

§ P < 0.01 when compared with 2-wk-old rats receiving same treatment.

<sup>||</sup> P < 0.005 when compared with 2-wk-old rats receiving same treatment.

(P < 0.025 compared with 20-mg-MMI group) and the same trend was shown by the 2-mg-MMI group. As shown in Fig. 1, many rats in these groups had elevated levels of T<sub>3</sub>. However, the lack of measurements of T<sub>4</sub>, T<sub>3</sub>, and TSH in the same samples precludes further analysis.

*Effect of different MMI regimens on neonatal Cx enzyme activities (Fig. 2).* The enzyme activities were measured in tissue samples from the same pups discussed above. At 2 wk, AT, S.D., and ATPase activities were significantly reduced only in the 20-mg-MMI group. At 3 wk, reduced levels of these three enzyme activities were observed in the 5-mg-MMI group as well, and at 4 wk one of the enzymes, AT, was also reduced in the 1-mg-MMI group. Although all these differences from the controls reached statistical significance, the values found in the hypothyroid pups were seldom <60% of the euthyroid controls. The difference observed at 2 wk between the 20-mg-MMI group and the controls, although modest, was quite reproducible, as is evident in Table V (see below) and from several other subsequent experiments not shown.

I 5'D activity responded quite differently to MMI. At 2 wk, both 5- and 20-mg-MMI groups showed a striking 10–13-fold increase in the enzyme activity measured with either substrate, and there was no difference between these two groups. The 1-mg-MMI group showed a modest increase significant only when the enzyme was assayed with rT<sub>3</sub> as substrate. At 3 and

4 wk both 5- and 20-mg-MMI groups also showed comparable increases, but the activity was only three- to fivefold that of controls, although at 4 wk we measured only rT<sub>3</sub> 5'-deiodination. The changes in 5'-deiodination of T<sub>4</sub> and PTU-insensitive rT<sub>3</sub> deiodination as assayed here were identical throughout, with the correlation coefficient of all pairs of such assays equal to 0.991.

*Correlation between serum thyroid hormones and the observed changes in the various enzyme activities (Figs. 3, 4A–C).* As indicated above, the various MMI

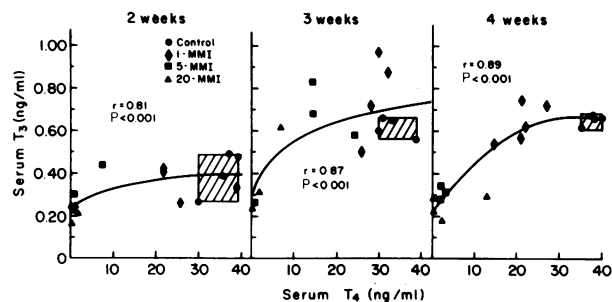


FIGURE 1 Serum T<sub>4</sub> and T<sub>3</sub> concentrations in neonatal rats with MMI-induced congenital hypothyroidism. MMI was given to the mothers in the drinking water from day 16 of gestation throughout the observation period. Each dot represents one individual rat and each symbol a different MMI concentration in milligrams/100 ml of drinking water. The square hatched area represents the control ranges for both variables. The coefficient of correlation corresponds to a power model.

TABLE II  
Serum TSH Concentrations in 2½–3-Wk-Old Neonatal Rats  
with MMI-induced Congenital Hypothyroidism

MMI*	n	TSH†
		μU/ml
Control	4	128±28
2	8	389±298
5	10	513±245‡
20	6	245±73 <sup>  </sup>

Data represent mean±SD.

Most of these rats were littermates of those used for experiments 1 (3 wk) and 2 in Table I.

\* MMI, methimazole concentration (mg/100 ml) in drinking water as described in Methods and Table I.

† TSH in microunits or RP1 standard.

‡  $P < 0.01$  vs. control.

<sup>||</sup>  $P < 0.025$  vs. 5-mg-MMI group.

doses induced a broad range of serum  $T_4$  and  $T_3$  concentrations, with great overlap in the responses to different doses of the drug. This allowed us to examine the relationships between serum thyroid hormones and enzyme activities rather than those between the latter and the doses of MMI. The correlation between I 5'D

(using  $rT_3$  as a substrate in the presence of 1 mM PTU) and serum levels of either  $T_4$  or  $T_3$  are shown in Fig. 3. A number of mathematical models for  $y = f(x)$  were tested and the one that consistently gave the best fit was a parabolic model, where  $y = a + bx + cx^2$ . The correlation between I 5'D and serum  $T_4$  is  $>0.96$  in all cases. Three aspects of these data are of interest: (a) the  $y$ -intercept decreased between 2 and 3 wk with no further change between 3 and 4 wk; (b) the slope of the regression line for serum  $T_4 < 30$  ng/ml is significantly higher at 2 wk than at 3 or 4 wk; and (c) except for 1 pup, no supranormal I 5'D value was found if serum  $T_4$  was normal. The correlation between I 5'D and serum  $T_3$  (Fig. 3) was not as high as with  $T_4$  ( $r = 0.66-0.92$ ). Of the three characteristics noted for the I 5'D- $T_4$  correlations, only the first two are true for serum  $T_3$ . Elevated I 5'D enzyme activity levels were observed in pups with normal or even elevated serum  $T_3$  concentrations.

The relationship of AT, S.D., and ATPase with serum  $T_4$  and  $T_3$  at each age is depicted in Fig. 4A-C. The parabolic model again gave the highest correlation coefficients. Although there were usually significant correlations between the AT and S.D., and serum levels of  $T_4$  and  $T_3$ , the variation of ATPase was hardly, if at all, explained by the variation of serum

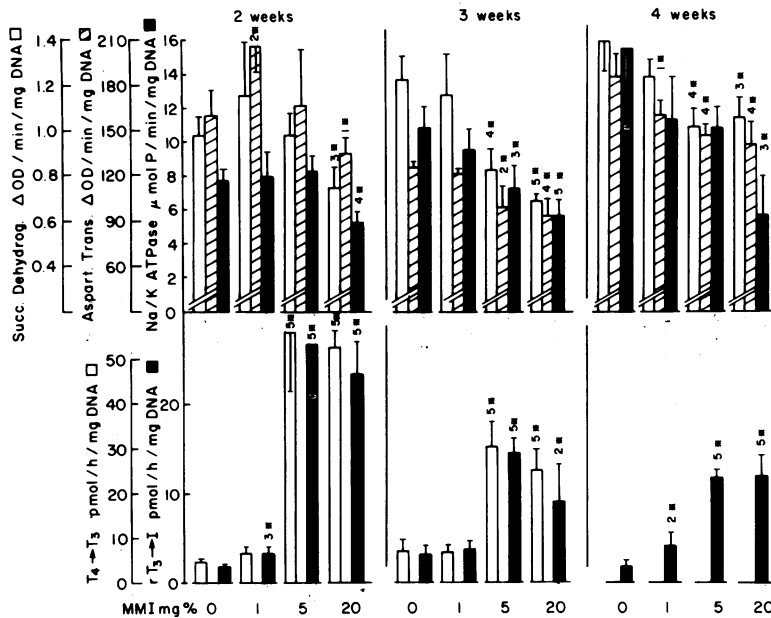


FIGURE 2 Effect of MMI-induced congenital hypothyroidism in various enzymes of the Cx of neonatal rats. Succ. Dehydrog., succinic dehydrogenase. These rats are the same as those whose serum values are shown in Table I, Exp. 1, and in Fig. 1. All enzyme activities were measured in crude homogenates as described in Methods.  $T_4 \rightarrow T_3 = T_4$  to  $T_3$  conversion as described in Methods.  $rT_3 \rightarrow I = {}^{125}I$  release from  $5[{}^{125}I]rT_3$  in the presence of 1 mM PTU as described in Methods. The brackets represent 1 SD. Statistical significance with respect to age-matched controls signaled by 1\* ( $P < 0.05$ ) to 5\* ( $P < 0.001$ ).

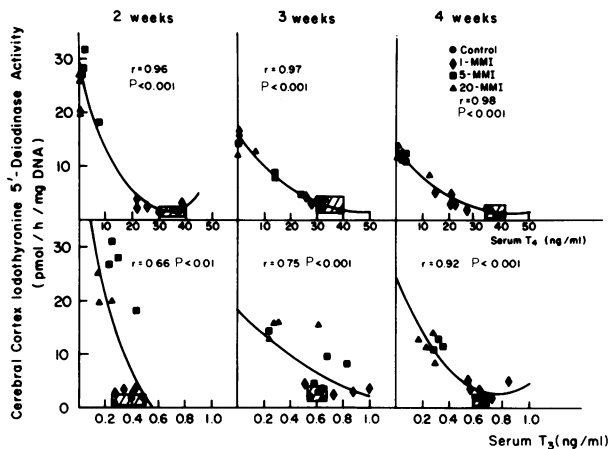


FIGURE 3 Relationship between I 5'D activity and serum  $T_4$  and  $T_3$  concentrations in neonatal rats with MMI-induced congenital hypothyroidism. Symbols and explanations as for Fig. 1. Correlation coefficients and regression lines correspond to a parabolic model  $y = a + bx + cx^2$  as described in Results.

thyroid hormone levels. The most relevant aspects of these analyses are (a) the correlation coefficients are lower than those observed for I 5'D, indicating that other variables are responsible for a significant amount of the enzyme variability; (b) at every time point the correlation coefficient was higher for serum  $T_4$  than for serum  $T_3$ ; and (c) a completely different pattern was apparent at 2 wk when compared with the observations at 3 and 4 wk. At 2 wk, normal or even elevated enzyme levels were observed with subnormal  $T_4$  levels (as low as 2.5 ng/ml), regardless of the serum  $T_3$  concentration, although at this age it was normal in one-third of the MMI-treated pups. At 3 wk, and especially 4 wk, normal values of AT or S.D. were infrequent among the MMI-treated pups. In pups of these ages, a normal serum  $T_4$  was a necessary, but not sufficient, condition for normal AT and S.D. activities, but normal values were observed independently of the serum  $T_3$  level.

*Comparison of  $T_4$  to  $T_3$  conversion activity response to hypothyroidism between Cx and liver (Fig. 5).* This experiment was done in 17-d-old rats. Mean serum  $T_4$  and  $T_3$  concentrations are shown in Table I, Exp. 2. Although there was a very high inverse correlation between the I 5'D activity in the Cx and serum  $T_4$ , comparable to that shown in Fig. 2, in the liver the correlation was in the opposite direction and not as high. In liver, normal I 5'D activities were observed in the 2- and 5-mg-MMI group in spite of a reduced serum  $T_4$ . These rats (diamonds and squares identified with numbers in Fig. 5) often had elevated levels of serum  $T_3$ , whereas those with decreased enzyme activity had both a low serum  $T_4$  and  $T_3$ . Consistent with

the data in Fig. 2, a reduced serum  $T_4$  was generally associated with an elevated I 5'D in the cortex. The regression line for  $T_3$  approaches the normal range of I 5'D when the serum  $T_3$  is well over the control range, i.e., between 0.6 and 0.8 ng/ml.

*In vivo generation of  $T_3$  from  $T_4$  in MMI-treated neonatal rats (Tables III and IV).* The data shown above suggest that the neonatal rat Cx has a high capacity to respond to hypothyroid levels of thyroid hormone by increasing I 5'D activity. This response could normalize the other cerebral enzymes in the face of markedly reduced serum  $T_4$  levels. The experiments shown in Tables III and IV were intended to determine whether the elevated I 5'D activity found in vitro was a reflection of the in vivo capacity of Cx to generate  $T_3$  from  $T_4$ . These experiments were performed in 2-wk-old pups, because at that time the response is maximal.

In the experiment shown in Table III, 600 ng of [ $^{131}$ I] $T_4$ /100 g bw, approximately one-third of the daily production rate of  $T_4$  for 2-wk-old rats (15), was given to euthyroid and 20-mg-MMI pups 16 h before death. IOP, an inhibitor of I 5'D, was also given to some pups to block in vivo  $T_3$  formation. The cortex:serum ratio of [ $^{131}$ I] $T_4$  was greater in the euthyroid than in the 20-mg-MMI pups ( $P < 0.001$ ), and in both it was significantly increased by IOP to similar values ( $P < 0.001$ ). The cortex:serum ratio for [ $^{131}$ I] $T_3$  was increased threefold in 20-mg-MMI pups relative to controls ( $P < 0.001$ ), and this difference was abolished by IOP. Because of these differences, the  $T_3$ : $T_4$  ratio in hypothyroid cortex was 10-fold that in euthyroid rats. This difference in  $T_3$ : $T_4$  ratio was also abolished by IOP. Using the specific activity of the injected  $T_4$ , the concentrations of  $T_4$  and  $T_3$  derived from the  $T_4$  dose were calculated. The tissue  $T_4$  concentration was threefold greater in the euthyroid controls than in the 20-mg-MMI group ( $P < 0.001$ ), and in both groups IOP treatment increased the concentration of  $T_4$  severalfold ( $P < 0.001$ ). Despite the reduced concentration of  $T_4$  in the cortex of the hypothyroid pups, the concentration of  $T_3$  derived from  $T_4$  [ $T_3(T_4)$ ] was 3.3-fold higher in the 20-mg-MMI rats than in the controls ( $P < 0.001$ ). This concentration of  $T_3$  is about three times the reported  $T_3$  concentration of 1.2 ng/g in adult euthyroid rat cerebral cortex (35). At the time of death, the serum concentration of  $T_4$  derived from the dose was  $21.4 \pm 1.2$  ng/ml in the control group and  $17 \pm 1.9$  ng/ml in the MMI-treated rats ( $P < 0.001$ ). However, these figures may be ~25% overestimated since they are based on serum TCA-precipitable- $^{131}$ I (see below).

In a second experiment we evaluated whether the  $T_3$  acutely generated from  $T_4$  would have a biological effect. IOP was used for the same purpose as in the previous experiment. Because in that experiment, the

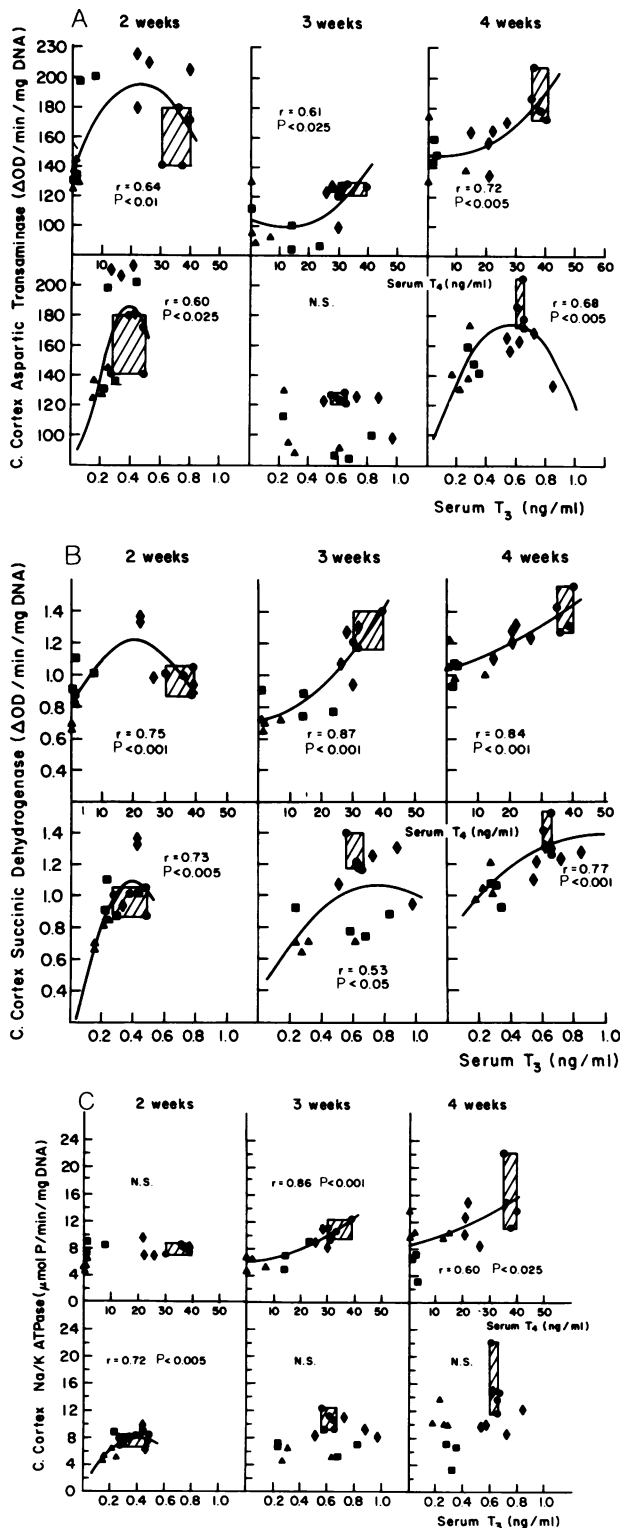


FIGURE 4 A-C. Relationship between Cx AT (A), S.D. (B), Na/K dependent ATPase (C), and serum T<sub>4</sub> and T<sub>3</sub> concentrations in neonatal rats with MMI-induced congenital hy-

amount of T<sub>3</sub> generated was severalfold the estimated adult concentration and in the IOP-injected pups a significant increase in cortex T<sub>4</sub> concentration occurred, we reduced the T<sub>4</sub> dose to 60 ng/pup, (150-300 ng/100 g bw), one-twelfth to one-sixth of the normal daily T<sub>4</sub> production rate for this age. The time interval after the T<sub>4</sub> injection was also increased to 24 h to allow expression of biological effects. Two 10-mg/100 g bw doses of IOP were injected 16 h before and just preceding the [<sup>125</sup>I]T<sub>4</sub>. Approximately 16 h before killing the pups, [<sup>131</sup>I]T<sub>3</sub> (5 ng) was injected subcutaneously to estimate the plasma [<sup>125</sup>I]T<sub>3</sub> contribution to tissue [<sup>125</sup>I]T<sub>3</sub> and the influence that differences in T<sub>3</sub> distribution and degradation might have had on the observed levels of [<sup>125</sup>I]T<sub>3</sub>.

The results of this experiment are shown in Tables IV and V. At 24 h only a minimal increase in serum T<sub>4</sub> of 2.6-2.7 ng/ml was observed in both euthyroid and MMI-treated rats. The increment in T<sub>4</sub> in the Cx of euthyroid rats was 0.34 ng/g, ~30% of the normal endogenous T<sub>4</sub> content (35, 36), but in the hypothyroid pups this increment was only 0.06 ng/g. This, and the large increments in cortex T<sub>4</sub> induced by IOP, indicate that the lower cortex:serum T<sub>4</sub> ratio in hypothyroid pups is due mainly to accelerated T<sub>4</sub> degradation in these animals. A similar phenomenon of less magnitude was observed in the liver. The T<sub>3</sub> derived from the T<sub>4</sub> in serum, cortex, and liver was significantly higher in hypothyroid animals and the difference exceeded that expected from the differences in the dose per unit weight. Due to differences in weight, the hypothyroid rats received a mean of 1.6 times more T<sub>4</sub> than the euthyroid pups, but the [<sup>125</sup>I]T<sub>3</sub> was 3.3, 2.5, and 4.6 times higher in the serum, liver, and cortex of the MMI-treated rats than in the euthyroid controls.

The serum [<sup>131</sup>I]T<sub>3</sub> in hypothyroid rats given T<sub>4</sub> was 0.23±0.03% of the dose per milliliter, whereas in the euthyroid controls it was 0.13±0.01% of the dose per milliliter (P < 0.001), suggesting a significant, albeit modest, decrease in T<sub>3</sub> clearance in the hypothyroid pups. If the 1.6-fold greater body weight of the control pups is taken into consideration, and assuming proportionality between body weight and volume of distribution, differences in fraction removal rate of T<sub>3</sub> do not turn out to be a major factor in the observed values of serum [<sup>131</sup>I]T<sub>3</sub> in the T<sub>4</sub>-treated hypothyroid pups. Furthermore, the cortex:serum ratio of [<sup>131</sup>I]T<sub>3</sub> was not different in any of these groups, indicating that there was no "sequestration" of T<sub>3</sub> in the nervous

pothyroidism. All three enzyme activities were measured in crude homogenates as described. Symbols and explanations are as for Fig. 1. Correlation coefficients and regression lines correspond to a parabolic model, as for Fig. 3. C. cortex, cerebral cortex.



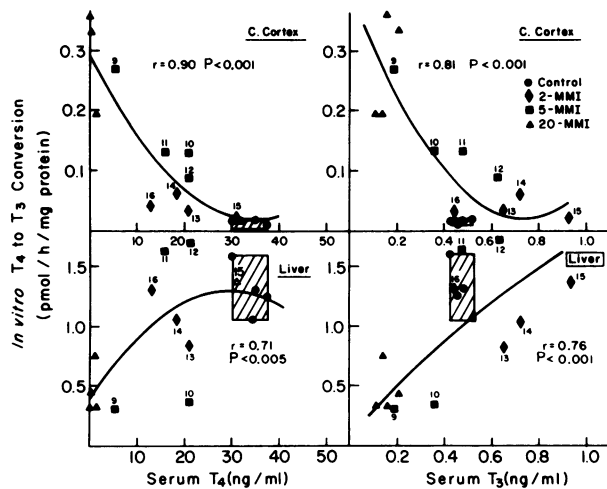


FIGURE 5 Relationship between Cx and liver I 5'D and the corresponding serum  $T_4$  and  $T_3$  concentrations in 2½-wk-old neonatal rats with MMI-induced congenital hypothyroidism. In both tissues enzyme activity was measured in crude homogenates as described in Methods. In both tissues, activity is expressed on a protein basis. Symbols and explanations are as for Fig. 1. Correlation coefficients and regression lines correspond to a parabolic model as for Fig. 3. The numbers by the symbols are used to identify each individual animal.

tissue. Using the injected  $[^{131}\text{I}]\text{T}_3$ , we estimated that, whereas 75% of the newly formed  $[^{125}\text{I}]\text{T}_3$  in both euthyroid and hypothyroid cortex was generated locally, in the liver virtually 100% was accounted for by the plasma  $[^{125}\text{I}]\text{T}_3$ . IOP administration markedly reduced  $T_4$  to  $T_3$  conversion and also decreased the degradation rate of  $T_3$  as judged by both tissue and serum concen-

trations of  $[^{131}\text{I}]\text{T}_3$ . In IOP-treated rats, all the  $[^{125}\text{I}]\text{T}_3$  found in both cortex and liver was accounted for by the plasma  $T_3$ , indicating that cortex  $T_4$  to  $T_3$  conversion was virtually abolished by this drug.

The enzyme activities (Table V) were reduced in all hypothyroid animals compared with the controls ( $P < 0.001$ ). AT was significantly higher in the 20-ng-MMI group treated with 60 ng of  $T_4$  than in any of the other groups of hypothyroid controls. This was the only enzyme that showed a significant increase after  $T_4$ . This response has been reproduced in several other experiments. IOP blocked this effect of  $T_4$  despite the marked increase in serum and tissue  $T_4$  that occurred in these animals (Table IV). This was not a nonspecific effect of IOP because it did not affect the level of the enzyme in hypothyroid pups receiving only tracer  $[^{125}\text{I}]\text{T}_4$  (3 ng). Liver  $\alpha$ -GPD also failed to increase with this small dose of  $T_4$ , which may be accounted for by the minimal elevation of serum  $T_3$  and the long latency of this enzyme to respond to thyroid hormone (37).

## DISCUSSION

We have recently described the mechanism of the pituitary response to a failing thyroid gland (reviewed in 13). In essence, the decreased serum  $T_4$  leads to pituitary hypothyroidism and the ensuing increase in TSH stimulates the thyroid to secrete  $T_3$ , keeping serum  $T_3$  within normal limits until well advanced thyroid failure. The results of the present experiments show that this mechanism is indeed operative in the neonatal rat, because  $T_3$  was maintained within normal limits in the face of markedly decreased (<10% of

TABLE III  
Effect of Thyroid Status on Cx  $T_3$  Concentration Generated 16 h after Injecting  
600 ng  $T_4$ /100 g bw to 2-Wk-Old Hypothyroid Rats

Condition	n	Treatment		Cx:Serum*			Iodothyronine in cortex§		
		$T_4$	IOP	$T_4$	$T_3(T_4)$	Cx $T_3/T_4$ †	$T_4$	$T_3$	
				$\times 10^5$			ng/mg DNA		
A	Eu	4	+	—	39±3	1.5±0.6	1.2±0.1	0.8±0.0	1.0±0.1
B	Eu	4	+	+	58±8	1.4±1.0	0.2±0.0	1.9±0.1	0.3±0.0
C	Hypo	4	+	—	17±5 <sup>  </sup>	4.3±0.3 <sup>  </sup>	13.0±3.8 <sup>  </sup>	0.3±0.1 <sup>  </sup>	3.3±0.1 <sup>  </sup>
D	Hypo	4	+	+	51±7	1.1±0.5	0.2±0.0	1.8±0.1	0.3±0.0

Data represent mean±SD.

Eu, euthyroid controls; Hypo, hypothyroidism induced by 20 mg/100 ml MMI in drinking water as described in Methods. IOP (10 mg/100 g bw) was injected intraperitoneally ~18 h before death, and  $[^{131}\text{I}]\text{T}_4$  (600 ng/100 g bw) was injected intravenously 2 h later.

\* Cx:Serum, cortex to serum ratio of  $[^{131}\text{I}]\text{T}_4$  or  $[^{131}\text{I}]\text{T}_3$  ( $T_4$ ) expressed as percentage dose per milligram DNA divided by percentage dose per milliliter serum.

† Cx  $T_3/T_4$ ,  $[^{131}\text{I}]\text{T}_3$  counts per minute  $[^{131}\text{I}]\text{T}_4$  counts per minute in paper chromatography of butanol extracts of Cx.

§ Calculated from the specific activity of the injected  $[^{131}\text{I}]\text{T}_4$ . To calculate the mass of  $T_3$ , the tissue radioactivity was multiplied as well by  $(2 \times [651/777])$  because of changes in specific activity and in molecular weight in the process of  $[^{131}\text{I}]\text{T}_4$  to  $[^{131}\text{I}]\text{T}_3$  conversion.

<sup>||</sup>  $P < 0.001$  with respect to A. For IOP, all  $P$  values < 0.001 with respect to A (Eu) or C (Hypo).

TABLE IV  
Effect of Thyroid Status on Serum, Cx, and Liver Thyroid Hormone Concentrations 24 h after an Intravenous Injection of 60 ng T<sub>4</sub> to 2-Wk-Old Rats

Treatment	Group		
	Euthyroid	Hypothyroid	
	A	B	C
	<i>mean±SD of n = 4/group</i>		
[ <sup>125</sup> I]T <sub>4</sub> (ng/100 g bw)*	159±7	263±27	286±11
[ <sup>131</sup> I]T <sub>3</sub> (ng/100 g bw)	13±1	22±2	24±1
IOP (mg/100 g bw)	—	—	10 × 2
Serum T <sub>4</sub> (ng/ml)	2.6±0.5	2.7±0.3	13.0±2.5 <sup>  </sup>
Serum T <sub>3</sub> (T <sub>4</sub> ) (ng/ml)	0.03±0.00	0.10±0.01‡	0.02±0.01 <sup>  </sup>
Serum [ <sup>131</sup> I]T <sub>3</sub> (% dose/ml)	0.13±0.01	0.23±0.03‡	1.80±0.47 <sup>  </sup>
Cx increment (ng/g)			
T <sub>4</sub>	0.34±0.06	0.06±0.01‡	1.02±0.21 <sup>  </sup>
Total T <sub>3</sub> (T <sub>4</sub> )	0.38±0.06	1.76±0.28‡	0.02±0.01 <sup>  </sup>
Local T <sub>3</sub> (T <sub>4</sub> )	0.28±0.06	1.29±0.21‡	0.00±0.00 <sup>  </sup>
Liver increment (ng/g)			
T <sub>4</sub>	1.47±0.19	0.95±0.05 <sup>¶</sup>	4.81±1.21 <sup>  </sup>
Total T <sub>3</sub> (T <sub>4</sub> )	0.14±0.01	0.35±0.04‡	0.10±0.06 <sup>  </sup>
Total T <sub>3</sub> (T <sub>4</sub> )	0.00±0.00	0.00±0.03	0.04±0.06
Tissue/serum [ <sup>131</sup> I]T <sub>3</sub> (ng/g÷ng/ml)			
Cortex	3.73±0.72	4.56±0.95	2.37±1.05
Liver	11.33±1.41	7.96±0.97§	4.76±0.96‡

\* The weight of the animals is given in Table V. IOP, 10 mg/100 g bw, was injected intraperitoneally 40 and 24 h before death. [<sup>125</sup>I]T<sub>4</sub> was injected intravenously 24 h and [<sup>131</sup>I]T<sub>3</sub> subcutaneously 16 h before killing the animals. [<sup>131</sup>I]T<sub>3</sub> was used to estimate the local production of [<sup>125</sup>I]T<sub>3</sub> by subtracting ((tissue/serum [<sup>131</sup>I]T<sub>3</sub>) × serum [<sup>125</sup>I]T<sub>3</sub>) from the total observed tissue [<sup>125</sup>I]T<sub>3</sub>. The gravimetric increments of T<sub>4</sub> and T<sub>3</sub> derived from the T<sub>4</sub> dose were calculated as described in Table III.

§ *P* < 0.01 with respect to A.

¶ *P* < 0.005 with respect to A.

‡ *P* < 0.001 with respect to A.

<sup>||</sup> *P* < 0.001 with respect to B.

normals) serum T<sub>4</sub> (Tables I and II, Fig. 1). Because of the elevation of TSH and because the Cx, as the anterior pituitary, derives most of the T<sub>3</sub> from local T<sub>4</sub> to T<sub>3</sub> conversion, one would expect that the Cx of pups with low serum T<sub>4</sub>—in spite of the normal serum T<sub>3</sub>—would show evidence of hypothyroidism. However, the present findings suggest that there are other mechanisms operative at cellular levels that can compensate for significant reductions in T<sub>4</sub> supply in the neonatal rat with partial thyroid impairment.

**MMI-induced congenital hypothyroidism.** The addition of graded concentrations of MMI to the drinking water provides a model that induces graded hypothyroidism with a wide spectrum of T<sub>4</sub> concentrations suitable for our purpose. In addition, this model better reflects the situation most commonly seen in humans in that athyreosis is a less common cause of

congenital hypothyroidism than is dysgenesis or ectopically located and poorly functioning thyroid tissue (38, 39). Because of the residual thyroid tissue in these infants, serum T<sub>4</sub> is variably reduced and often serum T<sub>3</sub> is normal. In another common cause of congenital thyroid impairment, iodine deficiency, normal cord serum T<sub>3</sub>, and low T<sub>4</sub> are common findings (40, 41). Therefore, the experimental model used in the present studies has distinct parallels to human disease.

The possibility that MMI, by a mechanism other than the well known antithyroid effect, may account for our findings is remote. In Methods we have presented evidence that the drug does not affect the enzyme assays nor can it reduce the enzyme levels in surgically thyroidectomized rats. The better correlation of our results with serum thyroid hormone levels than with the dose of MMI and the effect of T<sub>4</sub> treat-

TABLE V  
Effect of 60 ng of T<sub>4</sub> on Various Cerebral Cortex Enzymes and Hepatic α-GPD in 2-Wk-Old Hypothyroid Rats

Treatment	Group				
	Euthyroid	Hypothyroid			
	A	B	C	D	E
	<i>mean±SD; n = 4/group</i>				
[ <sup>125</sup> I]T <sub>4</sub> (ng)	60	60	60	3	3
[ <sup>131</sup> I]T <sub>3</sub> (ng)	5	5	5	5	5
IOP	—	—	+	—	+
Cx					
AT	130±10	88±6*	75±6	74±7	77±7
S.D.	13.6±1.0	8.9±1.7	9.4±0.5	8.7±0.7	8.7±0.8
Na/K ATPase	6.3±0.4	3.9±0.3	3.9±0.4	3.4±0.2	3.6±0.4
Liver					
α-GPD (×10 <sup>3</sup> )	33±4	14±4	16±1	12±2	14±2
Body weight (g)					
Basal	33±2	23±2	21±1	19±2	22±1
After	41±2	26±2	21±2‡	21±4	20±2‡
<i>P</i> (paired <i>t</i> )	<0.025	<0.001	NS	NS	<0.025

Groups A, B, and C are the same as shown in Table IV. All groups belong to the same experiment. The timing of T<sub>3</sub>, T<sub>4</sub>, and IOP are as described for Table IV.

When a *P* value is not indicated, it is either because it is NS or because it is not relevant. All hypothyroid *P* values were <0.001 with respect to A.

\* *P* < 0.05 with respect to C, D and E.

‡ *P* < 0.025 with respect to B.

AT, S.D., and α-GPD are expressed as optical density units per minute per milligram DNA (cortex), or per milligram of mitochondrial protein (liver). Na/K-dependent ATPase is expressed as micromoles P per minute per milligram DNA.

ment on the enzymes (Table V) in MMI-treated pups point in the same direction. In addition, the levels of all three enzymes have been shown to be reduced to an even greater extent in <sup>131</sup>I-induced neonatal hypothyroidism (4, 5). Although the thyroid hormone plasma levels were not given in those reports one would assume that <sup>131</sup>I can induce a more severe form of hypothyroidism. At the same time, those results (4, 5) indicate that qualitatively similar data are obtainable in hypothyroidism induced by other means.

*Effect of the various MMI doses on cerebral enzymes.* In spite of the reduced levels of serum T<sub>4</sub> in virtually all MMI-treated pups, at 2 wk only the 20-mg-MMI group showed biochemical evidence of hypothyroidism in the cortex with decreased AT, S.D., and ATPase (Fig. 2), whereas when the animals increased in age, and also in the duration of MMI exposure, modest tissue hypothyroidism was observed after exposure to lower quantities of MMI. In all these situations, the changes observed in these enzymes were modest, contrasting with the concomitant marked increases of the I 5'D in the MMI-treated animals. The 10-fold increase in I 5'D observed at 2 wk in the pups

receiving the highest doses of MMI compared with the three- to fivefold increase in the pups on the same MMI dose later on (Figs. 2, 3), and the relationship between the levels of AT, S.D., and ATPase and serum T<sub>4</sub> and T<sub>3</sub> at different ages, all suggest that the increased I 5'D activity in response to hypothyroidism is of high adaptive value. Furthermore, one can speculate that the success of such a mechanism in maintaining cerebral cortical euthyroidism will presumably depend on at least two, though perhaps more, factors. The first is the quantity of tissue substrate (T<sub>4</sub>) available and the second, the magnitude of the increase in I 5'D activity achievable. Thus, in the 2-wk-old rats, normal AT, S.D., and ATPase activities are seen in the 5-mg-MMI but the 20-mg-MMI animals show hypothyroid responses. Because the I 5'D is increased 10-fold in both, it would appear that inadequate quantities of T<sub>4</sub> are available in the latter. Although, on average, the serum T<sub>4</sub> was not statistically lower in 20-mg-MMI rats than in 5-mg-MMI at 2 wk (Table I), it tended to be higher in the latter (Fig. 1), and the experiments injecting low doses of T<sub>4</sub> indicate that minimal increments in serum T<sub>4</sub>, within the experimental

measurement error, could give rise to significant amounts of  $T_3$  in the cortex (see below). On the other hand, despite similar serum  $T_4$  concentrations at 3 and 4 wk, the 5- and 20-mg-MMI groups have subnormal cortical enzyme levels. It would appear that the three- to fivefold increases in I 5'D characteristic of these ages are insufficient to maintain the euthyroid state. The capacity for I 5'D to increase during hypothyroidism may be a function of age, as shown in Figs. 2 and 3, and is suggested by the fact that there is a spontaneous two- to threefold increase in cerebral I 5'D beginning at 10 d in euthyroid pups (8).

*Capacity of the Cx to generate significant amounts of  $T_3$  from comparatively small doses of  $T_4$ .* To support the hypothesis that the above mechanism could explain these results, it should be possible to demonstrate in vivo that the quantities of  $T_3$  provided to the cortex via 5'-deiodination of small amounts of  $T_4$  are quantitatively and physiologically significant. The results shown in Tables III-V demonstrate that this is the case. Thus, Table III shows that after injection of a small dose of [ $^{131}$ I] $T_4$ , the cortex:serum [ $^{131}$ I] $T_3$  ratio is threefold higher in hypothyroid rats than in euthyroid animals. The increased [ $^{131}$ I] $T_3$  in the cortex is not explained by increased concentrations of  $T_4$  in the cortex of the hypothyroid rats, because both serum  $T_4$  concentration and the cortex:serum ratio of  $T_4$  are actually lower in hypothyroid rats than in euthyroid animals. These data suggest rapid  $T_4$  to  $T_3$  conversion in the hypothyroid animal. Furthermore, the elevated  $T_3$ , both in absolute terms and relative to the  $T_4$  concentration seen in the hypothyroid neonate, is abolished by IOP. The results shown in Table IV add support to and expand the information provided by the above findings. In this experiment, the injected  $T_4$  dose was only 150–300 ng/100 g bw, and at 24 h this was associated with an insignificant increase in the serum  $T_4$ . The concomitant injection in these pups of [ $^{131}$ I] $T_3$  allowed estimates of the fraction of the tissue  $T_3$  increments that could be attributed to local  $T_4$  to  $T_3$  conversion using the approach that we have previously described (6, 12). Also, we could determine how much of the observed differences in tissue [ $^{125}$ I] $T_3$  ( $T_4$ ) can be accounted for by changes in  $T_3$  distribution and degradation. As would be expected from our studies in adult rats (17, 42), most of the [ $^{125}$ I] $T_3$  in the Cx could be attributed to  $T_4$  and  $T_3$  conversion occurring within the Cx in euthyroid pups. In both euthyroid and hypothyroid pups, 75% of the newly formed  $T_3$  ( $T_4$ ) found at 24 h in the Cx was produced locally. This may be disturbing at the first glance because the 10-fold increase in I 5'D would allow one to expect a greater relative contribution of the local source. We interpret the finding to be purely coincidental. Thus, these figures have been obtained 24 h after an intra-

venous pulse of  $T_4$ , and in Table IV it is apparent that the concentration of  $T_4$  has decreased markedly at that time in the cortex of these hypothyroid pups, i.e.,  $0.06 \pm 0.01$  ng/g compared with  $0.34 \pm 0.06$  ng/g in the controls. On the other hand, because the serum  $T_3$  ( $T_4$ ) was higher in hypothyroid pups by roughly the same factor as the total Cx  $T_3$  ( $T_4$ ) was, and because the tissue:plasma ratio of [ $^{131}$ I] $T_3$  was not different, the serum contribution was higher in hypothyroid pups than in the controls by the same factor as the tissue  $T_3$  ( $T_4$ ) was elevated. The relative contribution of local as compared with the serum source to cortex  $T_3$  found under these conditions does not represent the steady-state conditions where endogenous concentrations of  $T_4$  and  $T_3$  must be taken into consideration.

The higher serum  $T_3$  ( $T_4$ ) in hypothyroid pups may be due to two factors. One is decreased  $T_3$  degradation, as suggested by the higher concentration of [ $^{131}$ I] $T_3$ ,  $0.23 \pm 0.01\%$  of the dose per milliliter in hypothyroid pups as opposed to  $0.13 \pm 0.01\%$  in controls. However, because the hypothyroid animals were smaller by approximately the same factor, the difference in serum concentration is not readily accounted for by decreased fractional  $T_3$  degradation. Furthermore, assuming that the difference is totally due to reduced volume of distribution, the overall rate of serum  $T_4$  to  $T_3$  conversion is at least the same in hypothyroid neonatal rats as in euthyroid pups. At any rate, regardless of the factor(s) responsible for the higher serum  $T_3$  ( $T_4$ ) in the hypothyroid neonates, the cortex:serum ratio of [ $^{131}$ I] $T_3$  was not affected by the thyroid status, indicating that differences in  $T_3$  clearance are equally reflected in both compartments.

*Comparison between liver and cortex I 5'D and  $T_3$  generation.* In this context, it is interesting that the liver I 5'D showed a change in opposite direction to the cortex with hypothyroidism (Fig. 5). In agreement with this in vitro finding, the increased concentration of newly formed  $T_3$  ( $T_4$ ) found in this tissue in MMI-treated pups is accounted for by the observed higher serum  $T_3$  ( $T_4$ ). Two important implications can be derived from these findings: (a) the liver is not as important a source of  $T_3$  in neonatal hypothyroid rats as it is in the euthyroid adult rat or even the euthyroid neonate, and (b) the increased I 5'D observed in the cortex of the hypothyroid pups is physiologically relevant. Thus, in Table IV, had the cortex not undergone the observed increment in I 5'D, the elevation in serum  $T_3$  ( $T_4$ ) observed in the MMI-treated neonates would have resulted in an increment of only 0.75 ng  $T_3$ /g in the cortex, the sum of the serum contribution, 0.47 ng  $T_3$ /g, plus the local contribution of 0.28 ng  $T_3$ /g found in euthyroid rats. Instead, the actual increment, 1.76 ng  $T_3$ /g, was 2.3-fold greater.

In summary, in hypothyroid neonatal rats, mecha-

nisms other than the increase in TSH secretion and the ensuing augmented thyroidal T<sub>3</sub> secretion contribute to the maintenance of normal tissue T<sub>3</sub> concentrations. One of the mechanisms studied here in the cerebral cortex is a marked increase in fractional T<sub>4</sub> to T<sub>3</sub> conversion. This mechanism may be present in other tissues and even reflected in the serum because, in spite of the reduced liver I 5'D, there was at least an equal fraction of the T<sub>4</sub> converted to serum T<sub>3</sub> in hypothyroid pups when compared with their euthyroid counterpart. Lastly, this mechanism is more effective in some tissues such as Cx, where local T<sub>4</sub> to T<sub>3</sub> conversion is an important source of tissue T<sub>3</sub>, than in liver, where it is not (6, 12, 13, 17).

An important question raised by these findings is the signal that stimulates the increase in I 5'D, because based on the above hypothesis, one would assume that the cerebral cortical T<sub>3</sub> concentration is normal or near normal when this compensation has occurred. Fig. 3 demonstrates how closely related the activity of the I 5'D is to the concentration of T<sub>4</sub> in the serum and how poorly it relates to the level of serum T<sub>3</sub> despite the fact that there is an obvious relationship between concentrations of serum T<sub>3</sub> and T<sub>4</sub> in these MMI-treated pups (Fig. 1). The activity of the enzyme thus seems to be governed by the level of serum T<sub>4</sub> rather than by the T<sub>3</sub> generated from it. At present, we do not know how the enzyme is regulated by thyroid hormones, though other data from our laboratory indicate that this increase in activity is due to an increase in the maximum velocity ( $V_{max}$ ) as opposed to the Michaelis constant ( $K_m$ ) of the enzyme (10, 24). It is also not clear whether this  $V_{max}$  increase represents an activation of latent enzyme units or changes in the synthetic rate of the enzyme. It is possible, given the marked heterogeneity of cerebral cortical tissue, that cells responding to the change in T<sub>4</sub> with an increase in I 5'D are different from those that are reflected by the activities of AT, S.D., and ATPase. The possibility must also be raised that the enzyme is regulated by the level of T<sub>4</sub> directly, without the necessity for the generation of T<sub>3</sub> and the subsequent interaction of this T<sub>3</sub> with the nuclear receptor. Whatever the answer to the questions posed by these data, the findings suggest that the intracellular T<sub>3</sub> concentration is carefully regulated in the developing brain. It is most interesting that in the Cx the capacity to respond to hypothyroidism appears maximal during the time when the maturation rate is highest and the normal adaptive feedback regulation of the pituitary-thyroid axis is not completely developed (2, 3, 15).

In terms of the clinical implications of these findings, it would appear important to evaluate the neurological and psychological outcome of the congenitally hypothyroid infant in terms of the serum concentrations of T<sub>4</sub> rather than of T<sub>3</sub>. That the laudable

outcome of treatment of infants with congenital hypothyroidism discovered by screening is so much better than has been reported in the literature before the time of screening was begun (43) may be due to the fact that clinically detected congenital hypothyroidism is likely to be more severe than that discovered by the sensitive screening process. The data at 3 and 4 wk in these rats suggest that this compensatory mechanism cannot protect the Cx indefinitely. This is consistent with the fact that earlier intervention with thyroid hormone is consistently more successful in preventing irreversible mental retardation in the congenitally hypothyroid infant (44).

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