Effects of 3-Nitro-L-Tyrosine on Thyroid Function in the Rat: an Experimental Model for the Dehalogenase Defect

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ABSTRACT The effects on thyroid function of an inhibitor of tyrosine dehalogenase, 3-nitro-L-tyrosine (MNT) have been investigated in rats. In preliminary studies, marked inhibition of iodotyrosine deiodination was demonstrated in rats drinking 8 mM MNT. A series of experiments was then performed in which rats received Remington low iodine diet and 8 mM MNT as drinking fluid. This regimen had the following effects, compared to the effects of a low iodine diet alone: (a) a decrease in serum protein–bound iodine, elevation of serum thyrotropin level, goiter, and growth inhibition all prevented or reversed by iodine supplements; (b) on initiation of MNT, a 2- to 3-fold increase in the rate of release of radioiodine from the thyroid and concomitant urinary excretion of large amounts of organic iodine; and (c) after 2 wk of MNT, a greatly increased rate of thyroidal uptake and release of ^{131}I, an increase in the ratio of monoiodotyrosine-^{131}I to diiodotyrosine-^{131}I in thyroid proteolysates and the appearance of labeled iodotyrosines in serum.

Acute administration of MNT intraperitoneally to rats on either an iodine-deficient or iodine-sufficient diet did not inhibit thyroidal uptake of ^{131}I or alter the distribution of ^{131}I among thyroidal iodoamino acids.

It is concluded that MNT is an effective inhibitor of iodotyrosine deiodination in vivo, without other important actions on thyroid function. Thus, MNT treatment affords a model for the human dehalogenase defect. By provoking iodotyrosine secretion and consequent urinary loss of iodine, MNT can exaggerate the effects of a low iodine intake, producing goitrous hypothyroidism despite a rapid rate of iodine turnover in the thyroid.

INTRODUCTION

An enzymatic activity which catalyzes the removal of iodine or bromine from the 3 and 5 positions of L-tyrosine has been detected in various mammalian tissues, including thyroid, liver, and kidney (2, 3). In the thyroid, this "tyrosine dehalogenase" (iodotyrosine deiodinase, iodotyrosine deshalogenase, tyrosine deiodinase) is thought to have a special function, acting upon the iodotyrosines released during thyroglobulin hydrolysis and liberating iodide which can then reenter hormonogenetic pathways. Normally, deiodinating activity is so efficient that negligible amounts of iodotyrosines are secreted by the thyroid (4, 5).

These processes became of clinical concern when some patients with congenital goitrous hypothyroidism were described who were unable to deiodinate iodotyrosines, and who thus secreted iodotyrosines which were ultimately lost in the urine (6). It was postulated that this urinary loss of iodotyrosine iodine could produce an iodine deficiency so severe that hypothyroidism ensued. The reversal of hypothyroidism in several patients with the dehalogenase defect by treatment with iodine supplements (7-11) supports this postulate.

However, a question has been raised whether dehalogenase deficiency is a sufficient cause for hypothyroidism (12-14). Also, difficulties in studying human subjects have prevented a detailed analysis of iodine metabolism in the presence of dehalogenase deficiency, and the elucidation of what role, if any, iodotyrosine deiodination plays in the regulation of thyroid function when iodine intake is high. An experimental approach to these problems seemed possible with the recent description of compounds capable of inhibiting iodotyrosine deiodination in thyroid tissue in vitro and in rats in vivo (15). In the present study, the effects of treatment with a potent dehalogenase inhibitor, 3-nitro-L-tyrosine, on the thyroid function of rats were examined, to determine

TABLE I
Protocols for Chronic MNT Administration

Experiment No.	Duration	Group	Drinking fluid	^{131}I i.p.*	No. rats	Average initial weight
	days					g
1	7	A	Deionized water		8	120
		B	MNT, 8 mM		7	
		C	NaI, 5 mg/100 ml		8	
		D	NaI, 5 mg/100 ml + MNT, 8 mM		9	
2	11	A	Deionized water		6	190
		B	MNT, 8 mM		6	
		C	KI, 40 μg/100 ml		5	
		D	KI, 40 μg/100 ml + MNT, 8 mM		5	
		E	KI, 400 μg/100 ml		5	
		F	KI, 400 μg/100 ml + MNT, 8 mM		5	
3	21	A	Deionized water	9 days	4	150
		B	Deionized water, 15 days; MNT, 8 mM, 6 days	9 days	5	
4	21	A	Deionized water	4 hr	4	100
		B	Deionized water, 7 days; MNT, 8 mM, 14 days	4 hr	4	
5	21	A	Deionized water	1 or 4 hr	8	120
		B	Deionized water, 7 days; MNT, 8 mM, 14 days	1 or 4 hr	8	
		C	Deionized water (MNT i.p. on last day)	4 hr	4	

* Interval between ^{131}I administration and killing.

if this would provide a suitable animal model for the dehalogenase defect.

METHODS

Carrier-free Na^{131}I and Na^{125}I, and iodotyrosines labeled with ^{131}I were obtained commercially; 3-nitro-L-tyrosine (MNT)[1] was purchased from Cyclo Chemical Corporation, Los Angeles, California. Remington low iodine test diet (LID) was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, or General Biochemicals, Chagrin Falls, Ohio.

White male Wistar rats were used in all studies, and were given food (LID or Purina's Labena[2]) and water ad lib. until killed. When urine samples were desired, rats were kept in stainless steel metabolism cages; otherwise they were housed in group cages. In one study (experiment 5 described below) terminal urine specimens were obtained by ligating the urethra before killing and aspirating bladder contents at autopsy. Labeled compounds were administered intraperitoneally. Animals were killed by decapitation, and blood draining from the torso was collected; this method was employed to avoid possible effects of anesthesia on serum levels of TSH (16). Each thyroid was dissected free of fat and fascia, and weighed on a

[1] Abbreviations used in this paper: BDA, butanol:dioxane:2 N ammonia, 4:1:5; BuAc, butanol saturated with 2 N acetic acid; DIT, 3,5-diiodo-L-tyrosine; LID, Remington low iodine test diet; MIT, 3-iodo-L-tyrosine; MNT, 3-nitro-L-tyrosine; PBI, serum protein-bound iodine; T3, 3,5,3′-triiodo-L-thyronine; T4, L-thyroxine; TSH, thyrotropin.

[2] Ralston Purina Co., St. Louis, Mo.

torsion balance. When radioiodine had been given, the gland was then quickly frozen at − 60°C (Revco freezer[3]) and was later homogenized in 1 ml of a solution containing 0.11 M NaCl, 0.025 M 1-methyl-2-mercaptoimidazole, 0.007 M NaI, and 0.04 M Tris, pH 8.5; a sample was counted in an automatic gamma spectrometer, and 50 μl of pancreatin, 100 mg/ml, was added to the remainder which was then digested under toluene for 18 hr at 37°C.

To separate labeled compounds in serum, urine, and thyroid proteolysates, ascending paper chromatography was performed in BuAc or BDA. Radioactive zones were detected by radioautography and were then cut out and counted in an automatic gamma counter. Positions of added carrier amino acids were determined by spraying with ninhydrin, and added iodide was located by spraying with palladium chloride.

Radioimmunoassay for TSH (16) was performed by Doctors Robert D. Utiger and John F. Wilber; their assistance is gratefully acknowledged. PBI was measured by the Autotechnicon procedure at the Snodgras Laboratory, Department of Health and Hospitals, St. Louis, Mo.

In preliminary studies, urinary metabolites of MIT-^{131}I, DIT-^{131}I, and inorganic radioiodine in rats fed the regular Labena diet were analyzed by two-dimensional chromatography; see legends, Figs. 1-3. Five experiments were then performed to assess the effect of chronic administration of MNT on thyroid function; the protocols are summarized in Table I. MNT solutions were prepared by dissolving powder in a small amount of 2 N NaOH, then diluting with deionized water, and adjusting the pH to between 7 and 8 with 2 N HCl. In drinking solutions, the final concentration of MNT was 8 mM; also some drinking solu-

[3] Revco, Inc., West Columbia, S. C.
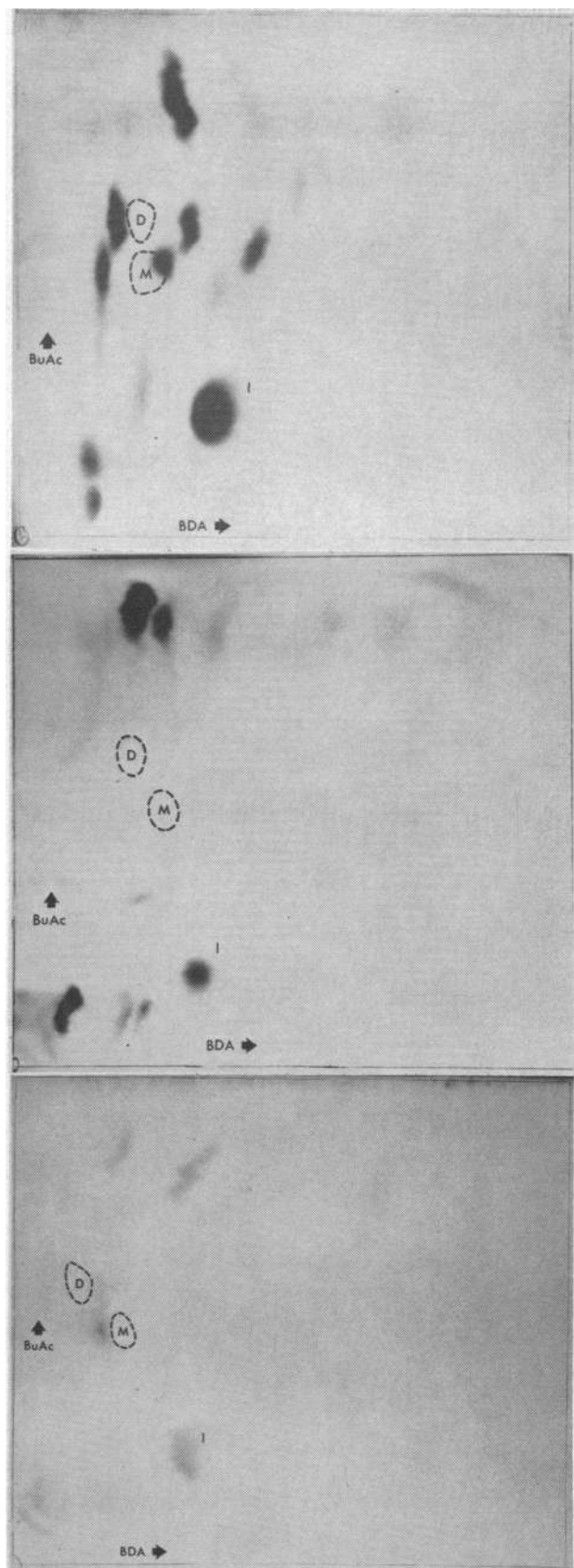
tions contained varying amount of KI or NaI (Table I). In experiment 3, carrier-free Na¹²⁵I was given 9 days before autopsy, and serial estimations of thyroidal radioactivity were made in vivo. Rats were lightly anesthetized with ether, and then were positioned 2 cm above a shielded scintillation probe so as to yield maximal counts on a rate meter; in this position, cpm were determined on a scaler, and compared with cpm in an aliquot of the injection solution, so that thyroidal radioactivity could be expressed as per cent of injected dose. Accuracy of this method was confirmed by comparing in vivo results just before killing with measurements of radioactivity in the thyroid removed at autopsy. In experiment 4, 4 hr radioiodine uptakes were determined following chronic MNT treatment. Experiment 5 was similar to experiment 4, except 1 hr uptakes were also measured in the control and chronic MNT groups, and 4 hr uptakes were measured in a third group, identical with the controls except injections of 0.1 M MNT, 0.25 ml intraperitoneally, were given 5 and 2 hr before killing.

An additional study, experiment 6, was done chiefly to assess effects of MNT on growth of rats receiving various iodine rations; the protocol is summarized in Fig. 11. In experiment 7, effects of acute administration of MNT on radioiodine uptake were studied. Details of this experiment are given in the results section.

Statistical methods employed included the *t* test for comparing small groups, analysis of variance, and the calculation of regression equations by the method of least squares (17). Unless otherwise noted, the index of variation cited is the SE.

RESULTS

Urinary metabolites of labeled MIT, DIT, and NaI. In rats receiving MNT, administration of labeled MIT or DIT was followed by the excretion of numerous labeled compounds; examples are depicted in Figs. 1 and 2. Similar compounds appeared in the urine of rats with labeled thyroid glands who were given MNT, as shown in Fig. 3. After administration of labeled MIT, DIT, or NaI to control rats, the predominant radioactive component in urine was inorganic iodide ($>90\%$ of radioiodine in each of serial urine samples collected for 4 days), although trace amounts of organic iodine compounds similar to those shown in Figs. 1–3 were occasionally seen. In no instance was a radioactive zone that corresponded exactly to an added DIT or MIT carrier identified with certainty in urine from either control or MNT-treated rats, although such compounds were later found in serum (see Fig. 9, below).

FIGURES 1–3 Urinary metabolites of iodotyrosines and of iodide in MNT-treated rats. Radioautographs of paper chromatograms developed in BuAc (first dimension) and BDA (second dimension). The origin is at the lower left of each figure; D, M, and I represent positions of added carrier DIT, MIT, and iodide, respectively. Before urine collection, rats had received: Fig. 1, MNT, 100 μmoles/100 g body weight and MIT-¹³¹I, 20 μCi intraperitoneally; Fig. 2, MNT, 100 μmoles/100 g body weight and DIT-¹³¹I, 20 μCi intraperitoneally; Fig. 3, MNT, 8 mM in drinking water, 48 hr after carrier-free Na¹²⁵I, 50 μCi intraperitoneally.

Effects of chronic MNT treatment on thyroid function.
In early studies it was found that injections of MNT, 25 µmoles intraperitoneally every 6 hr, could sustain a major degree of inhibition of iodotyrosine deiodination, and could accelerate goiter development in iodine-deficient rats. To provide a similar daily dose of MNT orally, 8 mM MNT was given as the drinking fluid; this produced 60-80% inhibition of deiodination of exogenous labeled DIT and MIT (i.e. 60-80% of radioactivity was recovered in urine in a form other than iodide) and caused the appearance of labeled iodotyrosine metabolites in the urine of rats given Na^{125}I (Fig. 3) or Na^{131}I. Thus, 8 mM MNT in drinking fluid became the standard regimen. In experiment 1, the effects of oral MNT were studied in rats receiving LID with and without large supplements of NaI (5 mg/100 ml) in drinking fluid (Table II). Rats receiving LID, MNT, and no iodine supplement had significantly larger thyroids and higher TSH levels than iodine-deficient controls; also, among rats providing sufficient serum for PBI determinations, the values were lower in the MNT-treated group. High iodine supplements, however, abolished the effect of MNT on thyroid weight and on TSH levels.

Experiment 2 was similar to experiment 1 except that lower levels of iodine supplementation (40 and 400 µg KI/100 ml) were tested, and LID was administered for a longer period (11 days instead of 7). Results are depicted in Fig. 4. Again, the iodine-deficient, MNT-treated rats had larger thyroids, higher TSH levels, and lower PBI's than iodine-deficient controls ($P < 0.01$
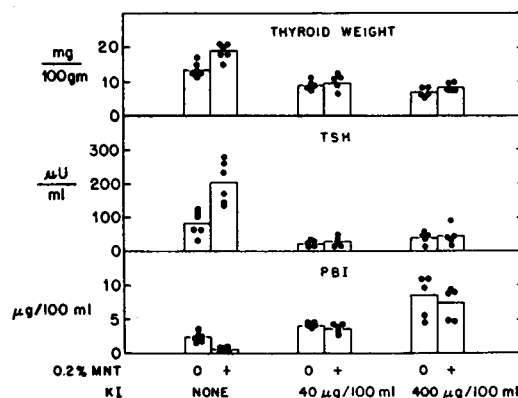

FIGURE 4 Effects of MNT on thyroid weight, serum TSH, and PBI (experiment 2). Rats received LID for 11 days; one-half also received 8 mM MNT (0.2 g/100 ml) in drinking water. As indicated at the bottom of the figure, some received KI, 400 µg/100 ml, and others, 40 µg/100 ml, in drinking water; a third group received no iodine supplement. Each symbol represents the value from an individual rat.

for all three indices). However, in both groups of iodine-supplemented rats MNT had no significant effects.[4]

Effect of MNT on thyroidal release of radioiodine.
In experiment 3, thyroidal radioiodine release rates were estimated in nine rats receiving LID; 66 hr after radioiodine injection, five rats were given 8 mM MNT as drinking fluid while the remainder continued on deionized water. The groups were matched, so that mean thyroidal ^{131}I content ±SE at 66 hr was 16.4 ±1.4% dose in controls and 15.9 ±2.6% dose in the MNT group. MNT caused an increased rate of release, as shown in Fig. 5. Expressed as a percent of the value at 66 hr, thyroidal radioactivity after 6 hr of MNT was significantly lower ($P < 0.01$ by t test) than in controls. For 100 hr after ^{131}I injection control rats appeared to have a logarithmic decline in thyroidal radioiodine, at a rate, estimated by the method of least squares, of 1.33%/hr. In the experimental group, the mean release rate before beginning MNT was 1.27%/hr, and it increased to

TABLE II

Effects of MNT, 8 mM in Drinking Water, on Thyroid Weight Serum TSH, and Serum PBI of Rats on LID (experiment 1)

		No iodine supplement		NaI, 5 mg/100 ml in drinking water	
Thyroid weight,					
mg/100 g body wt	No MNT	12.3 ±1.0	(8)*	8.0 ±1.1	(8)
	MNT	17.8 ±1.0	(7)	9.2 ±0.7	(9)
	P‡	<0.01		>0.10	
Serum TSH,					
µU/ml	No MNT	30.9 ±6.2	(8)	25.2 ±6.2	(8)
	MNT	149.4 ±29.2	(7)	16.4 ±1.9	(9)
	P	<0.002		>0.10	
Serum PBI,					
µg/100 ml	No MNT	4.2 ±1.0	(6)§		
	MNT	1.0 ±0.3	(6)§		
	P	<0.05			

* All values are given as mean ±SE, number of rats in parentheses.
‡ MNT and no MNT groups were compared by the *t* test.
§ Serum from two rats pooled for each determination; thus three determinations in each group.

[4] We later discovered, in confirmation of published reports (18), that inorganic iodide was not completely removed from serum by the method employed, shaking with Iobeads (Technicon Instruments Corp., Tarrytown, N. Y.). This should not affect PBI results in iodine-deficient rats or in those drinking 40 µg KI/100 ml, but could explain the somewhat high values in the rats receiving 400 µg KI/100 ml. A second potential problem is the inclusion of iodotyrosines and their metabolites in the PBI. Subsequent studies with equilibrium labeling have shown that iodotyrosines form no more than 10% of serum organic iodine and that iodothyronine levels are normal, in rats receiving MNT plus 200-1200 µg KI or NaI/100 ml of drinking fluid. The small proportion of iodotyrosines may reflect the earlier finding (15) that DIT disappears from serum quite rapidly even when its deiodination is inhibited.
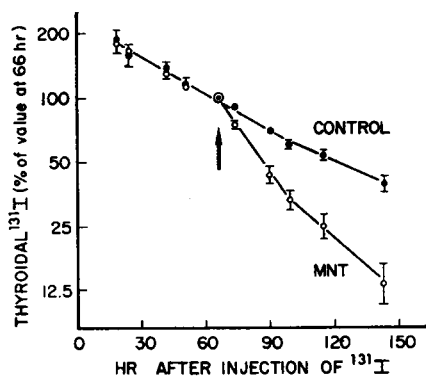
FIGURE 5 Effect of MNT on release of radioiodine from the thyroids of iodine-deficient rats (experiment 3). Carrier-free $Na^{131}I$ was injected intraperitoneally, 12 days after beginning LID. 3 days later, one group of rats received MNT, 8 mM in drinking fluid. Mean values for externally counted radioactivity in the thyroid region, ±SE, are shown for the four control rats (closed circles) and the five who received MNT (open circles). Where SE is not shown, it was less than the height of the symbol. Values were normalized by expressing each animal's thyroidal ^{131}I as a per cent of ^{131}I content at 66 hr, the time at which MNT was started.

3.30%/hr from the time of starting MNT until the 100 hr point. Acceleration of release was accompanied by an increased urinary excretion of radioiodine; ^{131}I in urines from MNT-treated rats, during the first 24 hr of treatment, was more than twice as great as in urines from controls (7.0 ±0.9% dose and 2.7 ±0.5% dose, respectively; by t test, $P < 0.01$) and was about 50% in organic form on chromatography in BuAc.

The animals of experiment 3 were killed 6 days after starting MNT treatment; the changes in thyroid weight (Table III) were similar to those seen in experiments 1 and 2.

Effects of chronic MNT treatment on radioiodine metabolism. In experiments 4 and 5, a regimen of MNT plus LID was given, with its usual effect on thyroid weight (Table III); ^{131}I was injected before killing.

TABLE III

Effects of MNT, 8 mM in Drinking Water, on Thyroid Weight and Serum PBI of Rats on LID

Experiment No.	Thyroid weight		
	Control	MNT	P^*
	mg/100 g body wt		
3	6.4 ±0.3(4)‡	10.4 ±0.8(5)	<0.001
4	10.4 ±1.1(4)	17.3 ±1.5(4)	<0.01
5	7.1 ±0.5(12)§	16.2 ±1.3(8)	<0.001

* Derived from t test.
‡ All values given as mean ±SE, number of rats in parentheses.
§ Includes 4 rats given MNT on day of killing.

Figs. 6 and 7 present results from Experiment 5 which included both a chronic MNT group, given 8 mM MNT for 14 days, and an acute MNT group, given injections of 25 μmoles MNT 5 and 2 hr before killing (see Methods). The chronic MNT group showed rapid accumulation of ^{131}I in the thyroid, to about 40% dose in 1 hr; then about half this ^{131}I was released by 4 hr. This did not represent uptake and discharge of inorganic iodide; thyroidal iodine was chiefly organic at both 1 and 4 hr (Fig. 7), and disappearance of ^{131}I from the thyroid was accompanied by the appearance of organic iodine in serum and in urine (Fig. 6). With regard to distribution of radioiodine among thyroidal amino acids, the chief alteration caused by chronic MNT treatment was an increase in the MIT/DIT ratio (Fig. 7). In experiment 4, only 4 hr uptakes were measured; the results obtained were similar to those of experiment 5 with respect to thyroidal ^{131}I content, chromatographic studies of thyroid proteolysates, and urine content of organic iodine. In neither experiment was the total iodothyronine radioactivity (per cent of thyroidal ^{131}I in the T3 + T4 zone following chromatography in BuAc) altered by MNT. However, in experiment 4, 2-dimensional chromatography (BuAc followed by BDA) revealed changes in the T3:T4 ratio; two control glands had ratios of 0.28 and 0.26, while two glands from MNT-treated animals had ratios of 1.41 and 1.36, $P < 0.05$ by t test. These 2-dimensional studies also failed to show any unusual radioiodinated compounds in the MNT group.

The relatively large amounts of ^{131}I in serum permitted characterization by paper chromatography. A 2-dimensional chromatogram of serum from an MNT-treated rat is shown in Fig. 8. Radioactive zones corresponding quite closely to the stable MIT, DIT, and T4 carriers are present, and unknown organic iodine compounds, of the type seen in urine, were not major components of serum radioactivity. Thus, 1-dimensional chromatography in BuAc seemed adequate to provide estimates of labeled iodotyrosine and iodothyronine concentrations. As sum-
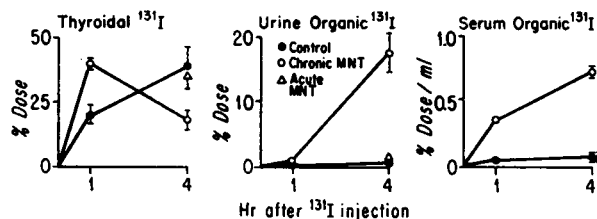

FIGURE 6 Metabolism of ^{131}I in rats receiving LID and MNT (experiment 5). Each symbol represents the mean value from four rats; SE's are shown, except when they were less than the height of the symbol. Control rats (●) were fed LID for 3 wk; chronic MNT rats (○) were given LID for 3 wk and 8 mM MNT for 2 wk; acute MNT rats (△) were fed LID for 3 wk and given 25 μmoles MNT intraperitoneally 5 and 2 hr before killing.

marized in Table IV, serums from the chronic MNT groups contained not only large amounts of iodotyrosines, but also larger amounts of iodothyronines than serums from the control groups. When MIT and DIT zones from the serum chromatograms of the chronic MNT groups were counted separately, the MIT:DIT ratios showed a fair correlation with the MIT-DIT ratios in thyroid proteolysates (Fig. 9). Despite the relatively large amounts of labeled iodotyrosines in serum, iodotyrosines could not be identified in urine. Urinary organic radioiodine was in the form of iodotyrosine metabolites similar to those shown in Figs. 1–3.

The rats given MNT acutely were quite similar to controls in most respects (Figs. 6 and 7) : they did have more radioactivity than controls in the iodotyrosine zones of serum chromatograms (Table IV) and a higher proportion of urinary ^{131}I in organic form, suggesting that iodotyrosine deiodination had been inhibited.

Growth of rats during chronic MNT treatment. The chronic MNT groups of experiments 4 and 5 fell progressively behind the other rats in weight gain; values from experiment 5 are shown in Fig. 10. This led to experiment 6, to test the effect of iodine supplements on MNT-induced growth failure. After 24 days of LID, rats were divided into three groups. Groups A and B both received iodine supplements in drinking fluid; the

FIGURE 8 Radioactive compounds in serum of an MNT-treated rat 4 hr after ^{131}I injection (experiment 4). The radioautograph of a paper chromatogram developed in BuAc (first dimension) and in BDA (second dimension) is shown. O = origin; *D, M, I, T4,* and *T3* represent positions of added carrier DIT, MIT, iodide, T4, and T3, respectively.

solution for group B also contained MNT, 8 mM. Group C received only MNT, 8 mM, for 8 days before iodine supplements were added. Within each group, two levels of iodine supplement were employed, 200 and 1200 μg NaI/100 ml; since no significant differences were associated with these different iodine intakes, the results are pooled in Fig. 11. When NaI was given together with MNT (group B), no inhibition of growth occurred. MNT alone caused growth inhibition, but later addition of iodine supplements restored the growth rate toward normal (group C). Average daily weight gains were subjected to analysis of variance. From days 1 to 24 (all rats on LID without MNT), and from day 34 to conclusion of the study (all rats on iodine supplement),
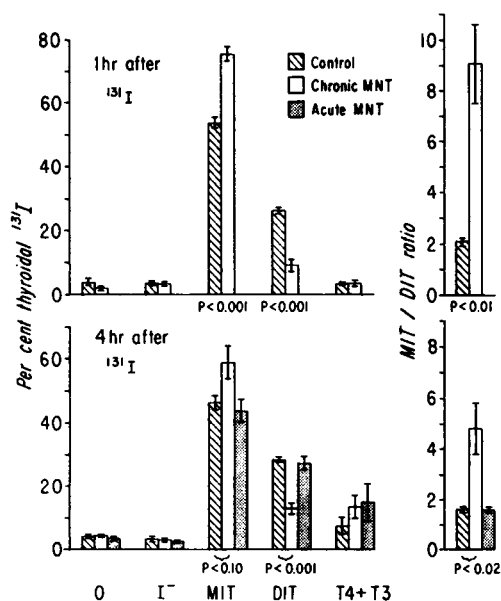

FIGURE 7 Chromatography in BuAc of thyroid hydrolysates from rats treated with MNT and LID (experiment 5). Each bar indicates the mean value from four rats, ±SE. Origin (O) includes all radioactivity up to the iodide zone. Control, chronic MNT, and acute MNT groups are as described in Fig. 6. Chronic and acute MNT groups were compared with the control group by *t* test; except where indicated, *P* values were 0.10 or greater.
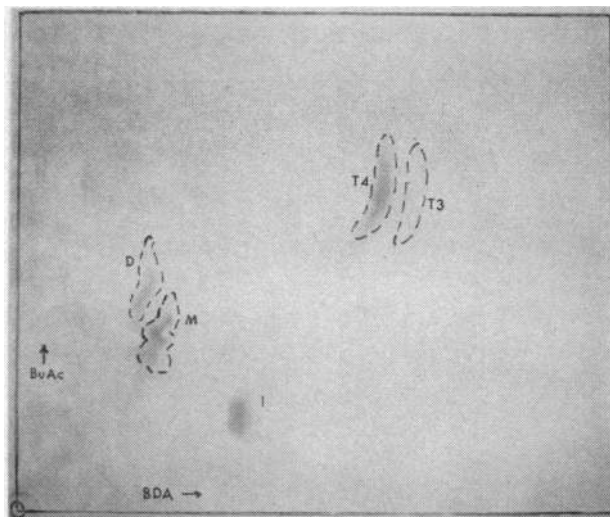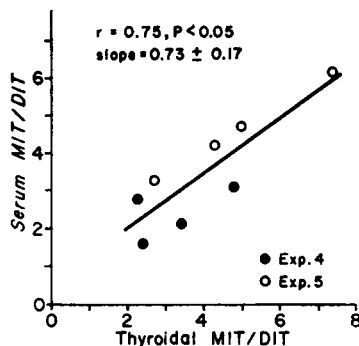

FIGURE 9 Correlation between MIT:DIT ratios in serum and in thyroid of MNT-treated rats. Each symbol represents an individual rat from the chronic MNT group of experiments 4 or 5, killed 4 hr after ^{131}I intraperitoneally.

TABLE IV
Effects of MNT + LID on [131]I Metabolism: Chromatography of Serum in BuAc

Experi-ment No.	Group	Total [131]I	[131]I in chromatographic zones			
			Origin	Iodide	MIT + DIT	T₃ + T₄
		% dose/ml	% dose/ml × 10³			
4	Control	0.600 ±0.110	27 ±6	54 ±7	33 ±2	486 ±111
	Chronic MNT	1.170 ±0.065	111 ±14	51 ±7	337 ±33	671 ±40
	P	<0.01	<0.01	NS	<0.001	NS
5	Control	0.215 ±0.049	6 ±1	162 ±58	8 ±1	38 ±25
	Chronic MNT	0.750 ±0.039	85 ±14	43 ±3	343 ±56	279 ±29
	P	<0.001	<0.01	NS	<0.01	<0.001
	Acute MNT	0.212 ±0.032	11 ±2	160 ±28	18 ±1	23 ±7
	P	NS	NS	NS	<0.001	NS

Each value is the mean ±SE from a group of four rats, killed 4 hr after ^{131}I injection.
P values are based on comparisons with the control group of the same experiment, using the t test; NS means P > 0.05.

weight gains did not differ significantly among the three groups. From days 24 to 34, weight gain was significantly less in group C (on MNT without iodine supplement through day 32) than in groups A and B, P < 0.001).

Acute effects of MNT on thyroidal ^{131}I uptake. In experiment 7, a more detailed study of the effects of acute administration of MNT was performed in rats receiving the regular Labena diet. As indicated in Fig. 12, a solution of MNT, or an equal volume of saline, was injected every 3 hr; radioiodine was given intraperitoneally 30 min after the first dose of MNT or saline. Thyroidal ^{131}I uptake at 2, 4, and 8 hr was not depressed by MNT.[5] Also, no significant differences were seen between control and MNT groups in distribution of radioactivity among iodoamino acids in thyroid proteolysates. Serum TSH was assayed in three of the animals from each group of nine; mean values ±SE were 25.2 ±3.9 μU/ml in controls and 20.9 ±4.2 μU/ml in MNT-treated animals.

Toxic effects of MNT. Some preliminary studies have been done to determine the toxicity of MNT. Single doses up to 200 μmoles (45 mg)/100 g body weight intraperitoneally had no discernible effects on rats; four doses of 100 μmoles/100 g at 2 hr intervals caused hypotonia

and lethargy, but the animals recovered. Serious ill effects were seen after 50 μmoles/100 g intraperitoneally every 3 hr for 2½ days; 4 of 13 rats so treated died, and the remainder showed marked lethargy, hypotonia, diminished food intake, and poor weight gain.

DISCUSSION

Prior studies have shown that MNT inhibits deiodination of iodotyrosines incubated with thyroid tissue slices or administered to rats in vivo (15). Also, 3,5-dinitro-L-tyrosine, which has inhibitory properties in vitro similar to those of MNT (15), causes secretion of iodotyrosines from prelabeled, perfused rat thyroid glands (20). In view of these findings, it was anticipated that MNT could inhibit deiodination of iodotyrosines formed in the thyroid gland in vivo. To test this assumption, the chromatographic studies of Figs. 1–3 were performed. These showed, first, that several metabolites could be formed from MIT and DIT during tyrosine dehalogenase inhibition; second, that similar organic iodine compounds were excreted following MNT administration to rats with radioiodine-labeled thyroids. The conclusion that MNT can provoke iodotyrosine secretion was later supported by identifying labeled MIT and DIT in the serum of MNT-treated rats, and showing that the MIT: DIT ratio in serum correlated with the ratio in the thyroid (Figs. 8 and 9).

The large number of urinary iodotyrosine metabolites found was somewhat unexpected, as was the failure to identify unchanged iodotyrosines in urine. In these respects, the MNT-treated rat differs from patients with the dehalogenase defect, who apparently do excrete unchanged iodotyrosines in their urine, and a smaller number of iodotyrosine metabolites (6, 7, 21). The difference may relate to the ability of the rat kidney to metabolize

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[5] In this experiment there was actually a mild stimulation of uptake by MNT at 2 and 4 hr, P < 0.05. One possible mechanism for such stimulation would be that proposed to explain increased iodide clearance after mannitol diuresis, namely, an autoregulatory response to lowered intrathyroidal iodide levels (19). However, stimulation of uptake has not been a constant finding following acute administration of MNT; e.g., ^{131}I uptake was not elevated in the acute MNT group of experiment 5, above. Resolution of this problem will require further investigation; nonetheless, the conclusion that MNT does not inhibit iodine uptake seems well supported by the data presented.

iodotyrosines rapidly by routes other than deiodination (22, 23), thus preventing their appearance in urine unchanged; even when rat serums contained large amounts of iodotyrosines, only metabolites were found in urine. In any case, the secretion of iodotyrosine iodine from the thyroid, followed by loss of this iodine in the urine, is a common feature of the dehalogenase defect and of MNT treatment, and could have consequences for thyroid function which are not modified by the more active nondeiodinative metabolism of iodotyrosines seen in the rat.

The goitrogenic potential of chronic MNT administration was assessed first, since goiter is the hallmark of the dehalogenase defect in man. In experiments 1 and 2, MNT plus iodine deficiency caused lower PBI's, higher TSH levels, and larger thyroid glands than did iodine deficiency alone. Also, these experiments demonstrated that iodine supplementation could prevent MNT from exerting its goitrogenic effect. This protective action was evident at a relatively low level of iodide supplementation, 40 μg KI/100 ml of drinking fluid, or an iodine intake of about 5 μg/day.[6]

According to current concepts (24), thyroid hormone (iodothyronine) secretion requires thyroglobulin proteolysis, which also liberates free iodotyrosines. The latter cannot be directly employed for hormone synthesis; however, the iodide derived from deiodination can be reutilized, and again incorporated into thyroprotein. Any compound which inhibits iodotyrosine deiodination should therefore prevent recycling of iodine, and cause accelerated loss of iodine from the gland. Experiment 3 showed that MNT could produce the expected acceleration of radioiodine release from the thyroid,

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[6] This level of iodine supplement may be near the threshold for prevention of MNT-induced goiter in rats. In a later experiment, which differed from experiment 2 in that rats were fed LID for 3½ wk, then given 40 μg NaI/100 ml, with or without 8 mM MNT, for 2½ wk, thyroids of the MNT group were larger, 22.3 ±2.2 mg, than those of the controls, 16.6 ±0.9 mg, $P < 0.05$.

FIGURE 11 Effect of MNT on weight gain of rats receiving LID and iodine supplements (experiment 6). Each symbol represents mean ±SE of 12 rats. Drinking solutions for groups A, B, and C are indicated at the bottom of the figure. MNT concentration was 8 mM; NaI concentrations were 200 μg/100 ml for six rats in each group and 1200 μg/100 ml for the remainder.

and further indicated that much of this released iodine was excreted in the urine as organic iodine. It seems evident that this depletion of preexisting thyroidal iodine stores could markedly increase the degree of iodine deficiency produced by LID.

Studies of radioiodine metabolism in iodine-deficient rats have shown that the rate of iodine accumulation, the ratio of MIT to DIT and of T3 to T4 in thyroid hydrolysates, and the rate of hormonal iodine secretion all undergo progressive increases during feeding of LID (25, 26). Experiments 4 and 5 showed that chronic MNT treatment exaggerated these effects of iodine deficiency, causing an increased rate of iodine
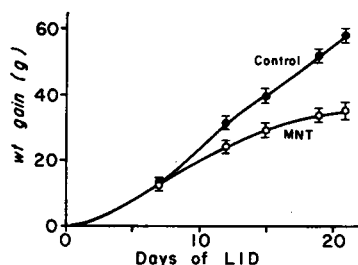
FIGURE 10. Effect of MNT on weight gain of rats receiving LID (experiment 5). The closed circles represent mean ±SE of 12 rats in the control and acute MNT groups; open circles represent mean ±SE of 8 rats which received MNT 8 mM in drinking fluid from day 7 until killing on day 21.
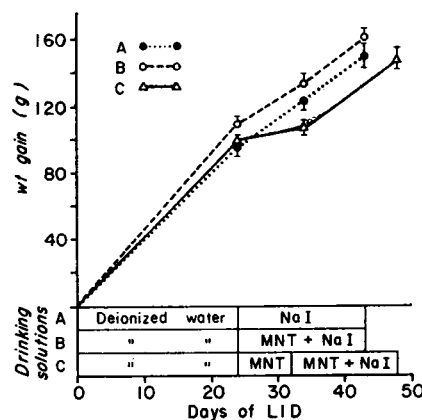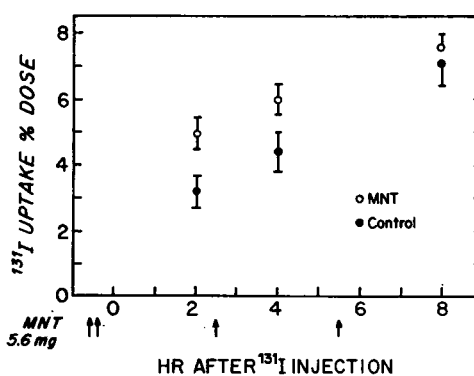
FIGURE 12 Effect of MNT on thyroidal ^{131}I uptake. Uptake of radioiodine by rat thyroids was determined 2, 4, and 8 hr after 10 μCi ^{131}I intraperitoneally. One-half the animals received 11.2 mg MNT (50 μmoles/100 g body wt) intraperitoneally 30 min before [131]I (as indicated by double arrows below figure), then 5.6 mg every 3 hr (indicated by single arrows). Each symbol indicates mean ±SE of nine rats.

accumulation, an increased MIT:DIT ratio, and an increased T3:T4 ratio. The rapid release of thyroidal iodine which occurred in the chronic MNT rats of experiment 5 may have partially reflected inhibition of recycling; however, iodine deficiency and TSH stimulation probably played a role, since the rate of release was much greater after a period of MNT treatment than it was immediately after beginning MNT administration (experiment 3, Fig. 5), and such rapid release did not occur in rats given MNT acutely. Analyses of serum radioactivity suggested that there was an increased rate of hormonal iodine release after chronic MNT treatment, although a significant difference from controls was shown only in experiment 5. Since PBI's were regularly depressed by chronic MNT treatment, the specific activity of hormonal iodine must have been greater in the chronic MNT groups, and it can be inferred that specific activity of iodine entering the thyroid was similarly elevated; thus absolute iodine uptake may have been low despite the high radioiodine uptake.

In experiments 4 and 5, we found MNT-treated rats gained weight less rapidly than controls, an effect not seen consistently in earlier studies. These experiments differed from earlier ones in that a period of LID administration preceded MNT treatment, and MNT was given for a longer period. Thus, it seemed possible that iodine deficiency played a role in MNT's effect on growth, and experiment 6 was performed to explore this possibility. The study showed, first, growth proceeded normally when supplemental iodine was given with MNT; second, after a long period of LID, a relatively short course of MNT could produce growth failure, and third, MNT-induced growth failure could be reversed by iodide. Combined with earlier evidence that MNT can depress PBI's and elevate TSH's, it seems probable that hypothyroidism is an important factor in the failure of weight gain produced by MNT.

Having shown that MNT is a goitrogen, the possibility was considered that it might promote goiter formation by mechanisms other than, or in addition to, tyrosine dehalogenase inhibition. It seemed particularly important to exclude inhibition of organic binding of iodide, an effect which is produced by a heterogeneous group of compounds, including phenols (27). Inhibitors of iodinations may produce effects similar to those of MNT, such as acceleration of radioiodine release (24, 28) and goiter formation antagonized by iodine (29, 30). Chronic administration of one such agent, aminoglutethimide, in doses which acutely cause partial inhibition, may lead to goiter with high iodine uptake (31). However, when given acutely MNT did not inhibit radioiodine uptake nor alter the distribution of radioiodine in thyroid hydrolysates, and is thus unlike

aminoglutethimide and other inhibitors of organic iodinations, which not only depress uptake acutely, but also increase the proportion of iodide and decrease the proportion of iodothyronines in the thyroid (31, 32). Another possible cause of goiter formation, direct stimulation of TSH secretion, was examined by determining serum TSH levels after acute MNT treatment, and no changes were detected.

To understand the mechanism by which tyrosine dehalogenase inhibition might produce goiter and hypothyroidism, some estimates of iodine balance may be relevant. Rats apparently cannot prevent the loss of organic iodine in the stools; on the Remington diet, fecal excretion accounts for about 50% of T4 and T3 turnover (33, 34), the remainder being deiodinated. Assuming a normal iodothyronine secretion of 1 μg I/day (24, 35), stool losses would almost equal iodine intake, which averages 0.5 μg/day on LID (26). The rat meets this problem in two ways: by markedly increasing thyroidal iodide clearance so that very little iodide is lost in the urine, and by secreting a higher proportion of T3, thus decreasing hormonal iodine needs (25). During tyrosine dehalogenase inhibition, organic iodine loss in the urine occurs and is related both to the rate of iodotyrosine liberation in the gland and to the degree of inhibition of deiodination. If the ratio between iodotyrosine liberation and iodothyronine secretion in terms of iodine is 2:1 (i.e. is similar to the ratio derived from stable iodine measurements in thyroid hydrolysates [24]), and tyrosine dehalogenase inhibition is such that 75% of the iodotyrosine iodine is lost in the urine, obligatory urine losses will be 1.5 times as great as iodothyronine iodine secretion; added to stool losses, twice as much iodine will be lost as is secreted as iodothyronine. With an iodine intake of 0.5 μg/day, a steady state would not be reached until iodothyronine secretion declined to 0.25 μg I/day, and maintaining this level would require an extreme thyroidal avidity for iodide. This series of events would provide an explanation for the changes in iodine metabolism seen after chronic therapy with MNT and LID: a great avidity for iodine, the recovery of a large proportion of secreted iodine in the urine, and lowered hormone levels despite glandular hyperactivity.

The usefulness of MNT and similar compounds in any experimental manipulations of thyroid function will be related to their specificity, i.e., to the ability to affect tyrosine dehalogenase alone without disturbing other functions either in the thyroid or in other organs. As discussed above, there is little evidence that MNT has direct effects on thyroidal iodine metabolism other than tyrosine dehalogenase inhibition. The available studies suggest that it is relatively inert in other metabolic pathways. In bacterial systems, MNT cannot

be activated and bound to RNA for protein synthesis, nor does it inhibit activation of tyrosine (36). Since iodotyrosines are potent inhibitors of tyrosine hydroxylase (37, 38), MNT could lead, via high tissue levels of MIT and DIT, to decreased catecholamine synthesis. This has been examined in rats treated with various regimens of MNT, and no changes in tissue catecholamine levels were found.[7] This suggests that iodotyrosine levels are not sufficiently elevated to affect tyrosine hydroxylase activity, and that MNT is not itself an inhibitor of this enzyme. In addition, using an assay system for tyrosine hydroxylase in which α-methyltyrosine is a potent inhibitor (39), no inhibition could be produced by α-methyl-3-nitro-L-tyrosine,[8] suggesting that the nitro group decreases affinity for the enzyme. Another pathway of tyrosine metabolism may be influenced by MNT, since a metabolite of MNT found in urine can inhibit homogentisic acid oxidase.[9] This phenomenon is under investigation, but appears remote from any changes in thyroid function.

It is concluded that marked inhibition of iodotyrosine deiodination in vivo can be produced by doses of MNT which are relatively nontoxic and which appear to produce few other biologic effects. Therefore, MNT affords a means of studying the consequences of tyrosine dehalogenase inhibition, and of constructing animal models for the human dehalogenase defect. The data presented here give evidence that dehalogenase deficiency is a cause of goiter and of hypothyroidism, confirming widely accepted hypotheses concerning the role of tyrosine dehalogenation in thyroid function and the mechanism of goitrogenesis in the dehalogenase defect. Thus, tyrosine dehalogenase inhibitors such as MNT form a new class of goitrogenic agents.

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[7] Benmiloud, M., and W. L. Green. Unpublished observations.

[8] Kuehl, F. A., Jr. Personal communication.

[9] Onstad, J., C. Dougherty, and W. L. Green. Unpublished observations.

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