

## Formation of diiodotyrosine from thyroxine. Ether-link cleavage, an alternate pathway of thyroxine metabolism.

A Balsam, F Sexton, M Borges, S H Ingbar

*J Clin Invest.* 1983;**72**(4):1234-1245. <https://doi.org/10.1172/JCI111079>.

### Research Article

Studies were performed to elucidate the nature of the pathway of hepatic thyroxine (T<sub>4</sub>) metabolism that is activated by inhibitors of liver catalase. For this purpose, the metabolism of T<sub>4</sub> in homogenates of rat liver was monitored with T<sub>4</sub> labeled with <sup>125</sup>I either at the 5'-position of the outer-ring (<sup>125</sup>I-beta-T<sub>4</sub>) or uniformly in both the outer and inner rings (<sup>125</sup>I-U-T<sub>4</sub>). In homogenates incubated with <sup>125</sup>I-beta-T<sub>4</sub> in an atmosphere of O<sub>2</sub>, the catalase inhibitor aminotriazole greatly enhanced T<sub>4</sub> degradation, promoting the formation of large proportions of <sup>125</sup>I-labeled iodide (<sup>125</sup>I-I<sup>-</sup>) and chromatographically immobile origin material (<sup>125</sup>I-OM), but only a minute proportion of <sup>125</sup>I-labeled 3,5,3'-triiodothyronine (<sup>125</sup>I-T<sub>3</sub>) (T<sub>3</sub> neogenesis). In an atmosphere of N<sub>2</sub>, in contrast, homogenates produced much larger proportions of <sup>125</sup>I-T<sub>3</sub>, and aminotriazole had no effect. In incubations with <sup>125</sup>I-U-T<sub>4</sub>, under aerobic conditions, control homogenates degraded T<sub>4</sub> slowly; formation of <sup>125</sup>I-labeled 3,5-diiodotyrosine (<sup>125</sup>I-DIT) was seen only occasionally and in minute proportions. However, in homogenates incubated under O<sub>2</sub>, but not N<sub>2</sub>, aminotriazole consistently elicited the formation of large proportions of <sup>125</sup>I-DIT, indicating that the ether link of T<sub>4</sub> was being cleaved by an O<sub>2</sub>-dependent process. Formation of <sup>125</sup>I-DIT in the presence of aminotriazole and O<sub>2</sub> was markedly inhibited by the substrates of peroxidase, aminoantipyrine, and guaiacol. GSH greatly attenuated the increase in DIT formation induced by aminotriazole, whereas the sulfhydryl inhibitor N-ethylmaleimide (NEM) [...]

**Find the latest version:**

<https://jci.me/111079/pdf>



# Formation of Diiodotyrosine from Thyroxine

## ETHER-LINK CLEAVAGE, AN ALTERNATE PATHWAY OF THYROXINE METABOLISM

ALAN BALSAM, FRANKLIN SEXTON, MARIETTA BORGES, and SIDNEY H. INGBAR,  
*Charles A. Dana Research Institute, Harvard-Thorndike Laboratory of Beth  
Israel Hospital, Department of Medicine, Beth Israel Hospital and Harvard  
Medical School, Boston, Massachusetts 02215*

**ABSTRACT** Studies were performed to elucidate the nature of the pathway of hepatic thyroxine ( $T_4$ ) metabolism that is activated by inhibitors of liver catalase. For this purpose, the metabolism of  $T_4$  in homogenates of rat liver was monitored with  $T_4$  labeled with  $^{125}\text{I}$  either at the 5'-position of the outer-ring ( $^{125}\text{I}-\beta-T_4$ ) or uniformly in both the outer and inner rings ( $^{125}\text{I}-\text{U}-T_4$ ). In homogenates incubated with  $^{125}\text{I}-\beta-T_4$  in an atmosphere of  $\text{O}_2$ , the catalase inhibitor aminotriazole greatly enhanced  $T_4$  degradation, promoting the formation of large proportions of  $^{125}\text{I}$ -labeled iodide ( $^{125}\text{I}-\text{I}^-$ ) and chromatographically immobile origin material ( $^{125}\text{I}-\text{OM}$ ), but only a minute proportion of  $^{125}\text{I}$ -labeled 3,5,3'-triiodothyronine ( $^{125}\text{I}-T_3$ ) ( $T_3$  neogenesis). In an atmosphere of  $\text{N}_2$ , in contrast, homogenates produced much larger proportions of  $^{125}\text{I}-T_3$ , and aminotriazole had no effect. In incubations with  $^{125}\text{I}-\text{U}-T_4$ , under aerobic conditions, control homogenates degraded  $T_4$  slowly; formation of  $^{125}\text{I}$ -labeled 3,5-diiodotyrosine ( $^{125}\text{I}-\text{DIT}$ ) was seen only occasionally and in minute proportions. However, in homogenates incubated under  $\text{O}_2$ , but not  $\text{N}_2$ , aminotriazole consistently elicited the formation of large proportions of  $^{125}\text{I}-\text{DIT}$ , indicating that the ether link of  $T_4$  was being cleaved by an  $\text{O}_2$ -dependent process.

Formation of  $^{125}\text{I}-\text{DIT}$  in the presence of aminotriazole and  $\text{O}_2$  was markedly inhibited by the substrates of peroxidase, aminoantipyrine, and guaiacol. GSH greatly attenuated the increase in DIT formation induced by aminotriazole, whereas the sulfhydryl inhibitor *N*-ethylmaleimide (NEM) activated the DIT-generating pathway, even in the absence of aminotriazole.

Activation of the in vitro formation of  $^{125}\text{I}-\text{DIT}$  from  $^{125}\text{I}-\text{U}-T_4$  was also produced by the in vivo administration of aminotriazole or bacterial endotoxin, an agent that reduces hepatic catalase activity. Studies with  $^{125}\text{I}-\text{DIT}$  as substrate revealed it to be rapidly deiodinated by liver homogenates under aerobic conditions. Recovery of  $^{125}\text{I}-\text{DIT}$  from  $^{125}\text{I}-\text{U}-T_4$  was increased by the addition of the inhibitor of iodotyrosine dehalogenase, 3,5-dinitrotyrosine. However, as judged from studies conducted in parallel with radioiodine-labeled DIT and  $^{125}\text{I}-\text{U}-T_4$  as substrates, none of the factors that altered the proportion of  $^{125}\text{I}-\text{DIT}$  found after incubations with  $^{125}\text{I}-\text{U}-T_4$  did so by altering the degradation of the  $^{125}\text{I}-\text{DIT}$  formed.

The factors that influenced DIT formation from  $T_4$  in rat liver had opposite effects on  $T_3$  neogenesis. Thus, aminotriazole, endotoxin, NEM, and an aerobic atmosphere, all of which enhanced DIT formation, were inhibitory to  $T_3$  neogenesis. In contrast, anaerobiosis and GSH inhibited ether-link cleavage of  $T_4$ , but facilitated  $T_3$  neogenesis.

The foregoing results suggest that a pathway for the ether-link cleavage of  $T_4$  to yield DIT is present in rat liver. Activity of this pathway, which appears to be peroxidase mediated, is inversely related to activity of the pathway for the  $T_3$  neogenesis. It is further suggested that this reciprocity reflects a reciprocal relationship between hepatic GSH and  $\text{H}_2\text{O}_2$ , the former increasing  $T_3$  formation and inhibiting DIT formation, and the latter producing opposite effects.

### INTRODUCTION

The major pathway for the peripheral metabolism of thyroxine ( $T_4$ )<sup>1</sup> in man and animals is reductive mon-

This work has been published in abstract form in 1979, *Clin. Res.*, 27:247A.

Received for publication 18 May 1982 and in revised form 3 May 1983.

<sup>1</sup> Abbreviations used in this paper: AAP, 4-aminoantipyrine, BAW, butanol/acetic acid/water, 12:3:5; DIT, 3,5-

odeiodination, whereby a hydrogen atom is exchanged for an iodine atom either at the 5'-position of the molecule to form 3,5,3'-triiodothyronine ( $T_3$ ) or at the 5-position to form 3,3',5'-triiodothyronine (reverse  $T_3$ ,  $rT_3$ ). It appears likely that these pathways result, respectively, in activation or inactivation of the hormone (1, 2).

Earlier studies, conducted before the importance of these specific pathways of  $T_4$  metabolism was appreciated, provided evidence that  $T_4$  could be deiodinated by tissue peroxidases (3). Thus, in vitro deiodination of  $T_4$  was induced in tissue-free systems by purified peroxidase plus  $H_2O_2$ , and was enhanced in liver homogenates by the in vitro or in vivo action of catalase inhibitors (4). In the present studies, we undertook to reexamine the products of the peroxidase-mediated pathway of  $T_4$  deiodination and to ascertain the relation of this pathway, if any, to the monodeiodination of  $T_4$  that leads to the generation of  $T_3$  ( $T_3$  neogenesis). The peroxidase-mediated pathway of deiodination was found to involve cleavage of the ether link of the  $T_4$  molecule, leading to the formation of 3,5-diiodotyrosine (DIT) from the amino acid portion of the molecule and to the rapid deiodination of the outer-ring residue. Activity of the pathway appeared to have a reciprocal relation to that of the 5'-monodeiodinating pathway that generates  $T_3$  (5).

## METHODS

**Animals.** Animals used were Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) that initially weighed 150–250 g. In each experiment, animals were closely matched with regard to weight, and unless otherwise stated were given standard pelleted rat chow and tap water ad lib.

**Materials.** Chemicals, reagents, and animal diets, as well as  $T_4$  and  $T_3$  labeled with  $^{125}I$  in their outer-ring ( $^{125}I$ - $\beta$ - $T_4$ ,  $^{125}I$ - $\beta$ - $T_3$ ), were obtained from commercial sources. Crystalline  $T_4$ ,  $T_3$ , DIT, 3-monoiodo-L-tyrosine (MIT), 3-amino-1,2,4-triazole (aminotriazole), 4-aminoantipyrine (AAP), guaiacol, *N*-ethylmaleimide (NEM), lactoperoxidase (60–80 U/mg), glucose oxidase (200 U/mg protein), catalase (8,000–20,000 U/mg protein) and glutathione (GSH) were purchased from Sigma Chemical Co. (St. Louis, MO). Endotoxin (lipopolysaccharide B; *Escherichia coli* 055:B5) was purchased from Difco Laboratories (Detroit, MI). Iodothyronines labeled with  $^{125}I$  in their phenolic rings,  $^{125}I$ - $\beta$ - $T_4$ , sp act 50–70 Ci/g and  $^{125}I$ - $\beta$ - $T_3$ , sp act 50–75 Ci/g were purchased from Abbott Diagnostics, Diagnostic Products (North Chicago, IL), and carrier-free  $Na^{125}I$  and  $Na^{131}I$  from New En-

gland Nuclear (Boston, MA). Pelleted laboratory chow, RMH 1000, was purchased from Agway-Country Foods, Agway Inc. (Syracuse, NY) and iodine-deficient chow from ICN Nutritional Biochemicals (Cleveland, OH).

**Preparation of uniformly labeled  $^{125}I$ -iodothyronines.**  $T_4$  and  $T_3$  labeled uniformly with  $^{125}I$  were prepared biosynthetically in rats given an iodine-deficient diet and distilled water for at least 2 wk. A dose of 5 mCi of carrier-free  $Na^{125}I$ -iodide was injected intraperitoneally, and rats were killed 4 h later. Thyroids were excised and homogenized in 0.5 ml Tris buffer, pH 7.4, containing 20 mM methimazole and 40 mM KI. The resulting homogenates were enriched with 5 mg Pronase and were incubated overnight at 37°C under  $N_2$  to assure maximum recovery of  $^{125}I$ -iodothyronines (6). Digests were then extracted with 1 ml butanol, and the extracts were completely evaporated under  $N_2$ . Extracted  $^{125}I$ -labeled compounds were dissolved in 100  $\mu$ l of methanol/2 N ammonia (3:1) and were separated one from another by descending paper chromatography in a hexane/tertiary amyl alcohol/2 N ammonia (1:10:11; HTA)-solvent system (7). Zones containing uniformly labeled  $^{125}I$ - $T_4$  ( $^{125}I$ -U- $T_4$ ) and  $^{125}I$ - $T_3$  ( $^{125}I$ -U- $T_3$ ) were identified by autoradiography, excised, and promptly eluted with methanol/ammonia. The eluates were evaporated under  $N_2$ , and  $^{125}I$ -U- $T_4$  and  $^{125}I$ -U- $T_3$  were then dissolved in 50% propylene glycol and stored at 0–4°C until used. Their purity was determined both by descending paper chromatography in HTA and by ascending chromatography in butanol/acetic acid/water (12:3:5; BAW).  $^{125}I$ -U- $T_4$  (sp act 2.2 mCi/ $\mu$ g)<sup>2</sup> was 90% pure and contained the following approximate proportions of  $^{125}I$ -labeled contaminants: iodide ( $I^-$ ), 8%; DIT, 2%; MIT, 1%; and  $T_3$ , 1.5%.  $^{125}I$ -U- $T_3$  (sp act 2.2 mCi/ $\mu$ g)<sup>2</sup> was ~90% pure and contained contaminants in the following approximate proportions:  $^{125}I$ - $I^-$ , 8%;  $^{125}I$ -DIT, 2%; and  $^{125}I$ -MIT, 0.5%.

**Preparation of radioiodine-labeled DIT.** DIT labeled with either  $^{125}I$  or  $^{131}I$  was prepared by a modification of the method of Sorimachi and Cahnmann (8). Iodination of MIT (6.25 nmol) with carrier-free  $Na^{125}I$  or  $Na^{131}I$  (2.0 mCi) was initiated by adding chloramine T (87.5 nmol) in 0.2 ml of 0.3 M phosphate buffer, pH 7.5, and was terminated 4 min later by adding sodium metabisulfite (87.5 nmol).  $^{125}I$ -DIT formed in the reaction mixture was then isolated by column chromatography on a cation-exchange resin, AG 50W-X4, according to a modification (9) of the method of Sorimachi and Ui (10). Approximately 80% of the radioactivity applied to the column was localized in a peak corresponding to DIT. The purity of the eluted peak was further assessed by paper chromatography in BAW. More than 90% of its radioactivity was in the form of  $^{125}I$ -DIT, with a small proportion (5%) of  $^{125}I$ - $I^-$  as its principal contaminant.

**Preparation and incubation of tissue homogenates.** Animals were killed by a blow to the head and their livers were rapidly excised. Portions of livers were weighed and, unless specified otherwise, were homogenized (1:9, wt/vol) in 0.05 M phosphate buffer, pH 7.4. Reaction mixtures were constituted of 2 ml of these homogenates, enriched as specified for each experiment with chemical additives and with one of the following radioiodine-labeled substrates:  $^{125}I$ - $\beta$ - $T_4$  (~1  $\mu$ Ci/ml, 0.020  $\mu$ g/ml);  $^{125}I$ -U- $T_4$  (0.25  $\mu$ Ci/ml), 0.11

diiodotyrosine; DNT, 3,5-dinitrotyrosine; HTA, hexane/tertiary amyl alcohol/2 N ammonia, 1:10:11;  $^{125}I$ - $\beta$ - $T_3$ , outer-ring labeled  $^{125}I$ - $T_3$ ;  $^{125}I$ - $\beta$ - $T_4$ , outer-ring labeled  $^{125}I$ - $T_4$ ;  $^{125}I$ -U- $T_3$ , uniformly labeled  $^{125}I$ - $T_3$ ;  $^{125}I$ -U- $T_4$ , uniformly labeled  $^{125}I$ - $T_4$ ; MIT, 3-monoiodo-L-tyrosine; NEM, *N*-ethylmaleimide; OM, chromatographically immobile origin material;  $rT_3$ , 3,3',5'-triiodothyronine;  $T_3$ , 3,5,3'-triiodothyronine;  $T_4$ , thyroxine.

<sup>2</sup> The specific activity of uniformly  $^{125}I$ -labeled  $T_3$  or  $T_4$  was calculated as the ratio of uniformly  $^{125}I$ -labeled  $T_4$  or  $T_3$  and stable  $T_4$  or  $T_3$ , measured, respectively, by competitive protein binding assay and radioimmunoassay.

ng/ml);  $^{125}\text{I}$ - $\beta$ - $\text{T}_3$  ( $\sim 1.3 \mu\text{Ci/ml}$ ,  $0.025 \mu\text{g/ml}$ );  $^{125}\text{I}$ -U- $\text{T}_3$  ( $0.25 \mu\text{Ci/ml}$ ,  $0.11 \text{ ng/ml}$ ); or DIT labeled with  $^{125}\text{I}$  or  $^{131}\text{I}$  ( $1.0 \mu\text{Ci/ml}$ ). Reaction vessels were continuously flushed with purified  $\text{O}_2$  or  $\text{N}_2$  and were incubated in a metabolic incubator at  $37^\circ\text{C}$  for 1 or 3 h. Parallel incubations were performed with nonmetabolizing control vessels containing buffer, labeled substrate, and chemical additives when appropriate, but no tissue. Reactions were terminated by placing incubation vessels in cracked ice. Portions of the reaction mixtures were then withdrawn, combined with outdated blood bank plasma (1:2, vol/vol) and stored frozen at  $-20^\circ\text{C}$  for subsequent analysis by paper chromatography or, in some instances, by cation-exchange column chromatography, as described above.

**Paper chromatography.** Reaction mixtures were routinely analyzed by unidimensional paper chromatography, in HTA or BAW systems, or both. HTA was most commonly used for studies of  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$  or  $^{125}\text{I}$ - $\beta$ - $\text{T}_3$  metabolism, and BAW for studies of  $^{125}\text{I}$ -U- $\text{T}_4$ ,  $^{125}\text{I}$ -U- $\text{T}_3$ , and labeled DIT metabolism. Radioiodine-labeled compounds were identified by staining of marker compounds and were quantitated by methods previously described (11).

**Statistical analysis.** Data were analyzed by the  $t$  test when the effect of a single experimental variable was examined, and by analysis of variance followed by Duncan's multiple range test when two or more experimental variables were studied (12).

## RESULTS

Since many varieties of experiments were performed, each type will be described individually in this section, together with the results obtained.

### *Aminotriazole in vitro: effect on the metabolism of*

$^{125}\text{I}$ - $\beta$ - $\text{T}_4$  (Table I A) and  $^{125}\text{I}$ - $\beta$ - $\text{T}_3$ . As judged from analyses in the HTA and BAW systems, whether incubated under  $\text{O}_2$  or  $\text{N}_2$ , control rat liver homogenates formed the following  $^{125}\text{I}$ -labeled products from  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$ :  $\text{T}_3$ ,  $\text{I}^-$ , and chromatographically immobile origin material (OM). Under  $\text{O}_2$ , enrichment with aminotriazole (60 mM) greatly enhanced both the degradation of  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$  and the formation  $^{125}\text{I}$ - $\text{I}^-$  and  $^{125}\text{I}$ -OM, but did not significantly affect the very slight net formation of  $^{125}\text{I}$ - $\text{T}_3$  that occurred under these conditions. Under  $\text{N}_2$ , formation of  $\text{T}_3$  was greatly increased, but aminotriazole was totally without effect. Formation of  $^{125}\text{I}$ - $\text{I}^-$  and  $^{125}\text{I}$ -OM was comparable when HTA or BAW was used for analysis, and  $^{125}\text{I}$ -DIT was not detected in either system.

Additional experiments were carried out to determine whether the very slight apparent formation of  $^{125}\text{I}$ - $\text{T}_3$  from  $^{125}\text{I}$ - $\text{T}_4$  in the presence of aminotriazole and  $\text{O}_2$ , despite the extensive deiodination of  $\text{T}_4$  that occurred under these conditions, could be explained by a very rapid degradation of  $\text{T}_3$ . For this purpose, the metabolism of either  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$  and of  $^{125}\text{I}$ - $\beta$ - $\text{T}_3$  by paired aliquots of liver homogenates was examined. In the presence of aminotriazole and  $\text{O}_2$ , the degradation of  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$  increased from  $5.5 \pm 0.8$  (mean  $\pm$  SE) to  $68.4 \pm 1.9\%$  ( $P < 0.001$ ), whereas the formation of  $^{125}\text{I}$ - $\text{T}_3$  was again not significantly changed ( $1.7 \pm 0.2$  vs.  $1.6 \pm 0.1\%$ ). In vessels incubated in parallel, the degradation of  $^{125}\text{I}$ - $\beta$ - $\text{T}_3$  was increased by aminotriazole

TABLE I  
The Effect of Aminotriazole on the Metabolism of  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$  in Rat Liver Homogenates

Treatment	Incubation atmosphere	n	$^{125}\text{I}$ - $\beta$ - $\text{T}_4$ degradation*	$^{125}\text{I}$ - $\text{T}_3$ formation	$^{125}\text{I}$ - $\text{I}^-$ formation	$^{125}\text{I}$ -OM formation
% added $\text{T}_4$						
A Aminotriazole in vitro†						
Control	$\text{O}_2$	3	$12.0 \pm 1.4$	$1.4 \pm 0.2$	$4.5 \pm 1.0\%$	$5.2 \pm 0.1$
Experimental	$\text{O}_2$	3	$79.4 \pm 14.6\%$	$2.1 \pm 0.4$	$57.4 \pm 12.7$	$18.8 \pm 2.8\%$
Control	$\text{N}_2$	3	$22.7 \pm 0.8$	$7.0 \pm 0.2$	$13.6 \pm 0.6$	$0.7 \pm 0.01$
Experimental	$\text{N}_2$	3	$20.7 \pm 0.9$	$7.2 \pm 0.6$	$11.7 \pm 1.0$	$0.8 \pm 0.1$
B Aminotriazole in vivo‡						
Control	$\text{O}_2$	4	$15.0 \pm 0.9$	$4.1 \pm 0.9$	$3.5 \pm 0.5$	$5.4 \pm 0.3$
Experimental	$\text{O}_2$	4	$95.8 \pm 0.3\%$	$2.3 \pm 0.8$	$78.9 \pm 0.6\%$	$14.4 \pm 0.3\%$
Control	$\text{N}_2$	7	$14.0 \pm 2.7$	$4.6 \pm 0.4$	$5.5 \pm 2.4$	$3.7 \pm 1.0$
Experimental	$\text{N}_2$	7	$11.8 \pm 1.4$	$2.8 \pm 0.2\%$	$5.4 \pm 1.3$	$2.0 \pm 0.2$

\* Values shown are mean  $\pm$  SE of those obtained by HTA for specimens from the number of animals indicated by  $n$ . Statistical analyses were performed using the  $t$  test.

† Aminotriazole (60 mM) was added to 10% homogenates of rat liver that were enriched with  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$  ( $1 \mu\text{Ci/ml}$ ,  $0.020 \mu\text{g/ml}$ ) and incubated in  $\text{O}_2$  or  $\text{N}_2$  for 3 h.

‡  $P < 0.02$  vs. corresponding control group.

§ Aminotriazole was administered to rats in two doses (0.4 g/100 g body wt i.p.) given 16 h apart and 10% homogenates of liver were prepared 4 h after the second injection. Homogenates were enriched with  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$  and were incubated in  $\text{O}_2$  or  $\text{N}_2$  for 3 h.

¶  $P < 0.001$  vs. corresponding control group.

from  $2.0 \pm 0.2$  to  $48.5 \pm 2.3\%$  ( $P < 0.001$ ). These data suggested that as much as half of the  $^{125}\text{I}$ - $\text{T}_3$  formed from  $^{125}\text{I}$ - $\text{T}_4$  in the presence of aminotriazole would have persisted. On this basis, it can be deduced that increased 5'-monodeiodination of  $\text{T}_4$  to yield  $\text{T}_3$  could have accounted for only a small fraction of the 12-fold increase in  $\text{T}_4$  deiodination that aminotriazole induced.

**Effect of aerobic preincubation with aminotriazole on  $\text{T}_3$  neogenesis (Fig. 1).** The foregoing experiments did not permit firm conclusions concerning the effect of aminotriazole on the generation of  $\text{T}_3$  from  $\text{T}_4$ , since aminotriazole was inactive under the anaerobic conditions that were optimal for the formation of  $\text{T}_3$ . Hence, studies were designed to allow the effects of aminotriazole and other additives to take place during a period of preincubation in  $\text{O}_2$ , while the metabolism of  $^{125}\text{I}$ - $\beta\text{-T}_4$  was allowed to take place during a subsequent period of incubation in  $\text{N}_2$ . Five experimental groups were used. Some aliquots of rat liver homogenate (25%, wt/vol) were not preincubated; others were variously enriched with aminotriazole (60 mM), GSH (5 mM), both, or neither, and were preincubated at  $37^\circ\text{C}$  under  $\text{O}_2$  for 5 min.  $^{125}\text{I}$ - $\beta\text{-T}_4$  was added to all specimens and they were then incubated under  $\text{N}_2$  for 3 h. Compared with homogenates not preincubated, homogenates preincubated at  $37^\circ\text{C}$  without additives formed much less  $^{125}\text{I}$ - $\text{T}_3$ . When the latter contained aminotriazole, further inhibition of  $^{125}\text{I}$ - $\text{T}_3$  formation was evident. Enrichment with GSH (5 mM) prevented the decrease in  $\text{T}_3$  neogenesis caused by preincubation at  $37^\circ\text{C}$ , and attenuated the inhibitory effect of aminotriazole.

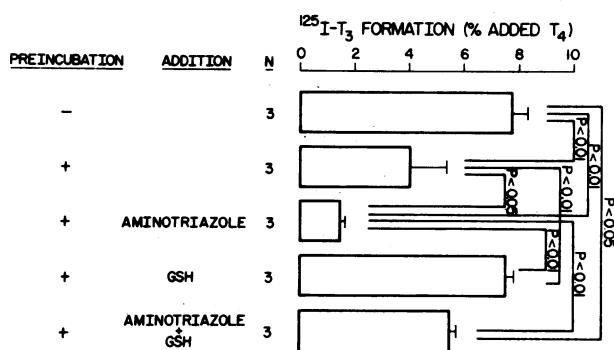


FIGURE 1 Homogenates of rat liver (1:3, wt/vol) were variously enriched with aminotriazole (60 mM) and GSH (5 mM) and preincubated at  $37^\circ\text{C}$  in  $\text{O}_2$  for 5 min. Control specimens were kept at  $0^\circ\text{C}$  (not preincubated) during this time.  $^{125}\text{I}$ - $\beta\text{-T}_4$  ( $1 \mu\text{Ci}/\text{ml}$ ,  $0.020 \mu\text{g}/\text{ml}$ ), was then added and specimens were incubated under  $\text{N}_2$  for 3 h to assess the formation of  $^{125}\text{I}$ - $\text{T}_3$ . In this and subsequent figures, values for the mean and SE, respectively, are depicted by horizontal bars and brackets, and  $n$  denotes the number of animals used.

**Aminotriazole in vivo: effects on the metabolism of  $^{125}\text{I}$ - $\beta\text{-T}_4$  (Table I B).** For these experiments, animals were given two injections of aminotriazole ( $0.4 \text{ g}/100 \text{ g body wt i.p.}$ ) 16 h apart and were killed 4 h after the second injection. Liver homogenates were prepared, enriched with  $^{125}\text{I}$ - $\beta\text{-T}_4$ , and incubated under  $\text{O}_2$  or  $\text{N}_2$  during the ensuing 3 h. In specimens incubated under  $\text{O}_2$ , prior administration of aminotriazole greatly increased the degradation of  $^{125}\text{I}$ - $\text{T}_4$  and the generation of  $^{125}\text{I}$ - $\text{I}^-$  and  $^{125}\text{I}$ -OM, and markedly decreased the percentage of  $^{125}\text{I}$ - $\text{T}_3$  generated from  $^{125}\text{I}$ - $\text{T}_4$ . In contrast, when homogenates from rats treated with aminotriazole were incubated under  $\text{N}_2$ , degradation of  $^{125}\text{I}$ - $\beta\text{-T}_4$  and formation of  $^{125}\text{I}$ - $\text{I}^-$  and  $^{125}\text{I}$ -OM were unchanged, whereas the formation of  $^{125}\text{I}$ - $\text{T}_3$  was markedly and significantly decreased.

**Aminotriazole in vitro: effects on the metabolism of  $^{125}\text{I}$ -U- $\text{T}_4$  and  $^{125}\text{I}$ -U- $\text{T}_3$ .** In ensuing studies, to permit the detection of metabolites bearing  $^{125}\text{I}$  derived from the inner ring of the  $\text{T}_4$  molecule,  $^{125}\text{I}$ -U- $\text{T}_4$  was used as the substrate (Table II A). Rat liver homogenates, containing either no additive or aminotriazole (60 mM), were enriched further with  $^{125}\text{I}$ -U- $\text{T}_4$  and incubated under either  $\text{O}_2$  or  $\text{N}_2$  for 1 h. Under these conditions, labeled products of  $^{125}\text{I}$ -U- $\text{T}_4$  metabolism included those found when  $^{125}\text{I}$ - $\beta\text{-T}_4$  was the substrate (i.e., OM,  $\text{I}^-$ , and  $\text{T}_3$ ); however, under certain circumstances  $^{125}\text{I}$ -DIT, but not  $^{125}\text{I}$ -MIT, was found. In control homogenates, whether incubated under  $\text{O}_2$  or  $\text{N}_2$ , formation of DIT was absent or negligible. However, under  $\text{O}_2$ , addition of aminotriazole led to the formation of readily detectable quantities of  $^{125}\text{I}$ -DIT ( $\sim 4\text{--}5\%$  of added  $^{125}\text{I}$ -U- $\text{T}_4$ ). No such effect of aminotriazole was seen under  $\text{N}_2$ . Results with respect to the degradation of  $^{125}\text{I}$ -U- $\text{T}_4$ , as well as the generation of labeled  $\text{I}^-$  and OM, were similar to those seen when  $^{125}\text{I}$ - $\beta\text{-T}_4$  served as substrate. As monitored with the HTA system,  $^{125}\text{I}$ - $\text{T}_3$  was routinely formed from  $^{125}\text{I}$ -U- $\text{T}_4$ , but its formation was not regularly measured, since the effects of various experimental manipulations on  $\text{T}_3$  neogenesis had been evaluated with  $^{125}\text{I}$ - $\beta\text{-T}_4$  as substrate.

Formation of  $^{125}\text{I}$ -DIT from  $^{125}\text{I}$ -U- $\text{T}_4$  during aerobic incubations with aminotriazole was also measured by means of anion-exchange column chromatography (Fig. 2), and the results obtained were verified by means of paper chromatography. For this purpose, control rat liver homogenates and others enriched with aminotriazole (60 mM) were incubated with  $^{125}\text{I}$ -U- $\text{T}_4$  under  $\text{O}_2$  or  $\text{N}_2$  for 1 h. Reaction mixtures were then extracted with 5 vol of ethanol; the extracts were evaporated completely under  $\text{N}_2$ , and their contents were dissolved in buffer. Before chromatography, specimens were further enriched with an extract of a Pronase hydrolysate of  $^{131}\text{I}$ -labeled thyroglobulin to provide

TABLE II  
The Effect of Aminotriazole on the Metabolism of  $^{125}\text{I}$ -U- $\text{T}_4$  by Rat Liver Homogenates

Treatment	Incubation atmosphere	n	$^{125}\text{I}$ -U- $\text{T}_4$ degradation*	$^{125}\text{I}$ -DIT formation	$^{125}\text{I}$ -I <sup>-</sup> formation	$^{125}\text{I}$ -OM formation
% added $\text{T}_4$						
A Aminotriazole in vitro†						
Control	O <sub>2</sub>	7	2.7±1.1	0.2±0	0.9±0.4	1.3±0.6
Experimental	O <sub>2</sub>	7	64.1±4.1§	4.1±0.7§	41.5±5.4§	15.0±4.0
Control	N <sub>2</sub>	3	6.4±3.3	0.2±0.1	3.0±0.7	2.5±3.5
Experimental	N <sub>2</sub>	3	2.4±1.3	0.2±0.2	2.1±1.1	0.2±0.2
B Aminotriazole in vivo¶						
Control	O <sub>2</sub>	4	4.1±0.6	0.5±0.2	2.1±0.6	1.5±0.1
Experimental	O <sub>2</sub>	4	74.0±1.0§	3.2±0.7	35.6±0.8§	29.8±0.6§
Control	N <sub>2</sub>	4	14.2±2.2	1.4±0.2	13.0±0.7	1.2±0.5
Experimental	N <sub>2</sub>	4	12.9±0.6	1.4±0.6	13.7±0.9	1.6±0.4

\* Values shown are mean±SE of those obtained in the BAW solvent system for specimens from the number of animals indicated by n. Statistical analyses were performed using the *t* test.

† Aminotriazole (60 mM) was added to 10% homogenates of rat liver that were then enriched with  $^{125}\text{I}$ -U- $\text{T}_4$  (0.25  $\mu\text{Ci}$ /ml, 0.11 ng/ml) and were incubated in O<sub>2</sub> or N<sub>2</sub> for 1 h.

§ *P* < 0.001 vs. corresponding control group.

|| *P* < 0.01 vs. corresponding control group.

¶ Aminotriazole was administered to rats in two doses (0.4 g/100 g body wt i.p.) given 16 h apart. Homogenates (1:9, wt/vol) of liver were prepared 4 h after the second injection, enriched with  $^{125}\text{I}$ -U- $\text{T}_4$ , and incubated in O<sub>2</sub> or N<sub>2</sub> for 3 h.

$^{131}\text{I}$ -DIT as a marker for any  $^{125}\text{I}$ -DIT formed from  $^{125}\text{I}$ -U- $\text{T}_4$ . The results of one of three closely agreeing experiments are depicted in Fig. 2. Contamination of the substrate by  $^{125}\text{I}$ -DIT was ~2% (upper panel). In homogenates that contained no aminotriazole, whether incubated under O<sub>2</sub> or N<sub>2</sub>, and in those enriched with aminotriazole but incubated under N<sub>2</sub>, the degradation of  $^{125}\text{I}$ -U- $\text{T}_4$  was slight, and the percentage of  $^{125}\text{I}$ -DIT found after incubation (2.0%) was similar to that which contaminated the substrate (data not shown). In contrast, in homogenates incubated aerobically with aminotriazole, greatly increased degradation of  $^{125}\text{I}$ -U- $\text{T}_4$  and increased proportions of  $^{125}\text{I}$ -DIT and  $^{125}\text{I}$ -I<sup>-</sup> were readily demonstrable (lower panel). The results in this and other experiments corresponded closely to measurements made in the same specimens by paper chromatography (data not shown).

To corroborate the finding that DIT was a product of aminotriazole-induced  $\text{T}_4$  metabolism, additional studies were carried out with 3,5-dinitrotyrosine (DNT), an inhibitor of iodotyrosine dehalogenase. In four experiments, the effect of aminotriazole (60 mM) was assessed in liver homogenates incubated in parallel with  $^{125}\text{I}$ -U- $\text{T}_4$  or  $^{125}\text{I}$ -DIT, to permit an evaluation of both DIT formation and degradation. In the absence of aminotriazole, DNT (10 mM) increased the apparent  $^{125}\text{I}$ -DIT formation from 0.8±0.5 to 1.9±1.1% (mean±SE). In the presence of the catalase inhibitor, DNT (10 mM) increased  $^{125}\text{I}$ -DIT formation from 3.7±0.5 to 5.7±0.7%.

$^{125}\text{I}$ -DIT metabolism was rapid in control homogenates, 56.6±13.4% of added DIT remaining after incubation. DNT inhibited DIT metabolism almost completely in control specimens (2.2%  $^{125}\text{I}$ -DIT added) and markedly, though less completely, in specimens enriched with aminotriazole (13.4±3.9%  $^{125}\text{I}$ -DIT added). These findings indicate that, as expected, inhibition of DIT metabolism within liver homogenates leads to increased accumulation of  $^{125}\text{I}$ -DIT from  $^{125}\text{I}$ -U- $\text{T}_4$  in the absence or the presence of aminotriazole. The effect of aminotriazole to increase DIT formation likely was underestimated, as inhibition of DIT degradation was less complete. The effects of aminotriazole and of DNT on the metabolism of  $^{125}\text{I}$ -U- $\text{T}_3$  were comparable to those seen when  $^{125}\text{I}$ -U- $\text{T}_4$  was substrate. In the presence of O<sub>2</sub>, aminotriazole greatly enhanced the degradation of  $^{125}\text{I}$ -U- $\text{T}_3$  (4.6±2.3 vs. 52.7±23%, *P* < 0.01), and increased the formation of  $^{125}\text{I}$ -DIT from 0.6±0.3 to 3.3±0.5%. DNT had little, if any, effect on apparent DIT formation in control specimens (0.8±0.1%), but in the presence of aminotriazole increased formation of DIT still further (5.9±0.3%).

**Aminotriazole in vivo: effects on the metabolism of  $^{125}\text{I}$ -U- $\text{T}_4$  (Table II B).** In these experiments, performed according to the protocol described above for studies with  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$ , liver homogenates from either control or aminotriazole-treated rats were incubated with  $^{125}\text{I}$ -U- $\text{T}_4$  under O<sub>2</sub> or N<sub>2</sub> for 3 h. Under O<sub>2</sub>, homogenates from animals given aminotriazole displayed marked increases in both the degradation of

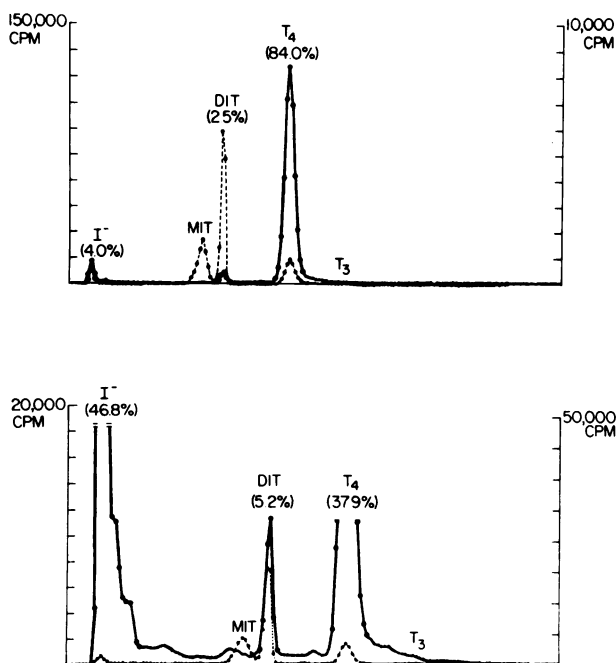


FIGURE 2 Homogenate of rat liver (1:9, wt/vol) was incubated with  $^{125}\text{I}$ -U- $\text{T}_4$  (0.25  $\mu\text{Ci}/\text{ml}$ , 0.11 ng/ml) and aminotriazole (60 mM) in  $\text{O}_2$  for 1 h and the reaction mixture was then extracted with 5 vol of ethanol. The nature of the extractable  $^{125}\text{I}$  was determined by cation-exchange column chromatography, with a pronase hydrolysate of  $^{131}\text{I}$ -thyroglobulin as a source of marker iodoamino acids. The scale for  $^{125}\text{I}$ -radioactivity is shown on the left and that for  $^{131}\text{I}$ -radioactivity is shown on the right. Representative chromatograms of the initial uniformly labeled  $^{125}\text{I}$ -U- $\text{T}_4$  substrate and the extract of the reaction mixture (rat liver homogenate incubated with aminotriazole), respectively, are shown in the upper and lower panels. Solid lines, substrate-derived  $^{125}\text{I}$ - $\text{I}^-$ ; dotted lines,  $^{131}\text{I}$ -labeled marker.

$^{125}\text{I}$ -U- $\text{T}_4$  and the formation of  $^{125}\text{I}$ - $\text{I}^-$  and  $^{125}\text{I}$ -OM. As in the *in vitro* studies, administration of aminotriazole led to the formation of increased quantities of  $^{125}\text{I}$ -DIT. These effects of aminotriazole were not seen when specimens were incubated under  $\text{N}_2$ .

**Effects of peroxidase substrates (Table III).** To evaluate the possibility that the formation of DIT from  $\text{T}_4$  is mediated by a peroxidase, we examined the effects of two substrates of peroxidase, AAP and guaiacol (13), on aminotriazole-induced DIT formation. As in earlier experiments, aminotriazole (60 mM) was added to aliquots of liver homogenates that were incubated with either  $^{125}\text{I}$ -U- $\text{T}_4$  or  $^{125}\text{I}$ -DIT under  $\text{O}_2$  for 1 h. In these experiments too, aminotriazole produced marked stimulation of both the metabolism of  $^{125}\text{I}$ -U- $\text{T}_4$  and the formation therefrom of  $^{125}\text{I}$ -DIT. Neither AAP (1 mM) nor guaiacol (1 mM) had an effect on  $\text{T}_4$  metabolism in the absence of aminotriazole, but both greatly

decreased the stimulation of DIT formation produced by aminotriazole. The inhibitory effects of AAP and guaiacol on the quantity of DIT formed from  $\text{T}_4$  could not be attributed to an enhancement of DIT degradation, since, to the contrary, these agents decreased the degradation of DIT in the presence of aminotriazole.

**Effect of GSH (Table IV).** Further evidence that peroxidase has a role in the formation of DIT from  $\text{T}_4$  was sought in experiments that tested the effects of adding GSH (5 mM), a cofactor for the reduction of  $\text{H}_2\text{O}_2$  by GSH peroxidase (14). Here, liver homogenates were enriched with aminotriazole (60 mM) and GSH (5 mM), separately or together, and were incubated with either  $^{125}\text{I}$ -U- $\text{T}_4$  or  $^{131}\text{I}$ -DIT under  $\text{O}_2$  for 1 h. As was evident in earlier experiments, aminotriazole activated degradation of  $^{125}\text{I}$ -U- $\text{T}_4$ , enhancing the formation of  $^{125}\text{I}$ -DIT,  $^{125}\text{I}$ - $\text{I}^-$ , and  $^{125}\text{I}$ -OM. Addition of GSH completely prevented this effect of aminotriazole. Again, the inhibitory effect of GSH on the formation of DIT was not attributable to increased DIT degradation. To the contrary, here, as in earlier experiments, the degradation of  $^{131}\text{I}$ -DIT was slightly accelerated in the presence of aminotriazole, and GSH counteracted this effect.

**Effects of NEM.** The ability of GSH to prevent the effect of aminotriazole suggested that under basal conditions the pathway of  $\text{T}_4$  metabolism in rat liver that is activated by aminotriazole might be tonically suppressed by GSH. Hence, experiments were performed to test the effect of the sulfhydryl inhibitor NEM on the metabolism of  $^{125}\text{I}$ -U- $\text{T}_4$  or  $^{125}\text{I}$ -DIT in homogenates incubated under  $\text{O}_2$  for 3 h (Fig. 3). As expected, addition of NEM (10 mM) produced a marked stimulation of the degradation of  $^{125}\text{I}$ -U- $\text{T}_4$ , accompanied by a corresponding increase in the formation of  $^{125}\text{I}$ -DIT,  $^{125}\text{I}$ - $\text{I}^-$ , and  $^{125}\text{I}$ -OM, without affecting the degradation of  $^{125}\text{I}$ -DIT. These effects of NEM were not seen in incubations carried out under  $\text{N}_2$ .

The effect of NEM on  $\text{T}_3$  neogenesis was assessed in liver homogenates from four rats incubated with  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$  under  $\text{N}_2$  for 3 h. Under these conditions, enrichment with NEM (10 mM) decreased the degradation of  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$  from  $17.0 \pm 1.7$  to  $12.0 \pm 0.7\%$  ( $P < 0.05$ ),  $^{125}\text{I}$ - $\text{T}_3$  formation from  $5.6 \pm 0.5$  to  $2.6 \pm 0.4\%$  ( $P < 0.005$ ), and  $^{125}\text{I}$ - $\text{I}^-$  formation from  $10.2 \pm 1.2$  to  $3.9 \pm 0.5\%$  ( $P < 0.005$ ).

**Effects of endotoxin (Fig. 4).** Overall deiodination of  $\text{T}_4$  in the intact rat and in homogenates of rat liver is markedly stimulated by the *in vivo* administration of bacterial endotoxin (4), an effect that has been ascribed to the attendant decrease in the hepatic concentration of catalase. In view of this, experiments were conducted with liver homogenates from rats injected intraperitoneally with endotoxin, 0.6 mg/100

TABLE III  
Effects of the Peroxidase Substrates AAP and Guaiacol on Aminotriazole-stimulated Metabolism of  $^{125}\text{I}$ -U-T<sub>4</sub> in Rat Liver Homogenates

Experimental group*	Addition*	n†	$^{125}\text{I}$ -U-T <sub>4</sub> degradation	$^{125}\text{I}$ -DIT formation	$^{125}\text{I}$ -I <sup>-</sup> formation‡	$^{125}\text{I}$ -OM formation	$^{125}\text{I}$ -DIT degradation
% added substrate							
a Control	—	9	4.3±0.8§	0.7±0.2	3.0±0.8	0.8±0.2	52.4±7.4
b Aminotriazole	—	9	46.3±4.8	2.9±0.2	26.1±3.0	17.0±2.5	73.0±6.4
c Control	AAP	9	4.2±1.1	0.9±0.3	3.0±1.0	0.6±0.2	50.9±9.8
d Aminotriazole	AAP	9	8.4±0.6	2.0±0.1	4.9±1.2	1.8±0.4	48.7±8.3
e Control	Guaiacol	7	4.5±0.8	0.7±0.2	2.9±0.7	0.7±0.2	62.1±7.4
f Aminotriazole	Guaiacol	7	13.3±1.6	1.2±0.2	6.0±1.0	5.8±1.3	47.2±6.2
Significant differences <sup>  </sup>			$P < 0.05$ a vs. f c vs. f e vs. f	$P < 0.05$ d vs. f  $P < 0.01$	$P < 0.01$ a vs. b b vs. c b vs. d b vs. e b vs. f	$P < 0.05$ d vs. f e vs. f  $P < 0.01$	$P < 0.05$ b vs. f
			$P < 0.01$ a vs. b a vs. d b vs. c b vs. d b vs. e b vs. f	a vs. b a vs. d b vs. c b vs. d b vs. e b vs. f c vs. d d vs. e	b vs. f	a vs. b a vs. f b vs. c b vs. d b vs. e b vs. f c vs. f	

\* Aminotriazole (60 mM), AAP (1 mM), and guaiacol (1 mM) were variously added to 10% homogenates of rat liver, which were then enriched with  $^{125}\text{I}$ -U-T<sub>4</sub> (0.25  $\mu\text{Ci}/\text{ml}$ , 0.11 ng/ml) or  $^{131}\text{I}$ -DIT (1  $\mu\text{Ci}/\text{ml}$ ) and incubated in O<sub>2</sub> for 1 h.

† n represents number of separate homogenates in which the metabolism of  $^{125}\text{I}$ -U-T<sub>4</sub> was studied. For experiments with  $^{131}\text{I}$ -DIT, n = 4.

§ Values shown are the mean±SE of those obtained in the BAW solvent system.

<sup>||</sup> Statistical analyses were performed using analysis of variance and Duncan's multiple range test (12).

TABLE IV  
Effects of GSH on Aminotriazole-stimulated Metabolism of  $^{125}\text{I}$ -U-T<sub>4</sub> in Rat Liver Homogenates

Experimental group*	Addition*	$^{125}\text{I}$ -U-T <sub>4</sub> degradation	$^{125}\text{I}$ -DIT formation	$^{125}\text{I}$ -I <sup>-</sup> formation	$^{125}\text{I}$ -OM formation	$^{125}\text{I}$ -DIT degradation
% added substrate						
a Control	—	8.2±5.9	0.1±0.1	2.0±0.7	6.7±5.3	56.6±2.4
b Aminotriazole	—	70.9±6.3	4.0±0.3	34.7±4.7	31.4±5.0	64.2±3.5
c Control	GSH	17.9±13.3	0.3±0.2	8.8±8.8	9.6±5.6	55.5±3.8
d Aminotriazole	GSH	14.6±7.8	0.1±0.1	5.2±4.3	10.1±5.6	40.0±5.5
Significant differences‡		$P < 0.01$ a vs. b b vs. c b vs. d	$P < 0.01$ a vs. b b vs. c b vs. d	$P < 0.01$ a vs. b b vs. c b vs. d	$P < 0.05$ a vs. b  $P < 0.01$ c vs. d	$P < 0.05$ a vs. b b vs. d

\* Aminotriazole (60 mM) and GSH (5 mM) were variously added to 10% homogenates of rat liver that were enriched with  $^{125}\text{I}$ -U-T<sub>4</sub> (0.25  $\mu\text{Ci}/\text{ml}$ , 0.11 ng/ml) or  $^{125}\text{I}$ -DIT (1  $\mu\text{Ci}/\text{ml}$ ) and then incubated in O<sub>2</sub> for 1 h.

‡ Values shown are mean±SE of those obtained in the BAW solvent study in experiments with four separate homogenates. Statistical analyses were performed by analysis of variance and Duncan's multiple range test.



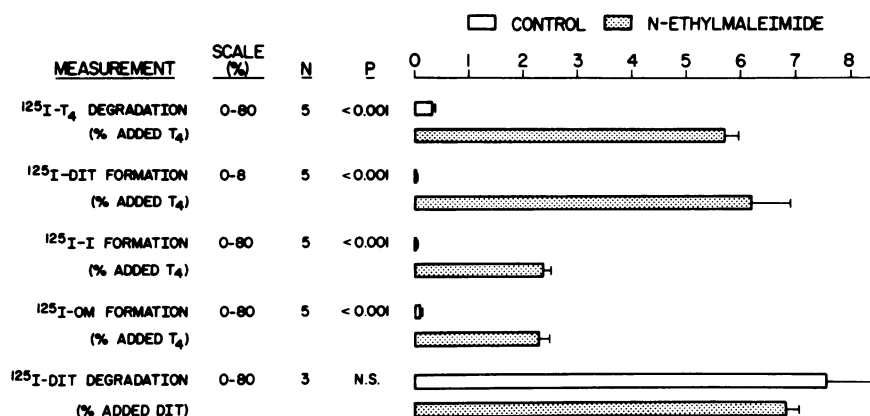


FIGURE 3 Homogenates of rat liver (1:9, wt/vol) were variously enriched with NEM (10 mM), <sup>125</sup>I-U-T<sub>4</sub> (0.25 μCi/ml, 0.11 ng/ml) or <sup>125</sup>I-DIT (1 μCi/ml) and incubated in O<sub>2</sub> for 1 h.

g body wt, 4 h before they were killed. Homogenates were incubated with <sup>125</sup>I-U-T<sub>4</sub> or <sup>125</sup>I-DIT under O<sub>2</sub> or N<sub>2</sub> for 3 h. In specimens from endotoxin-treated rats incubated aerobically, but not in those incubated under N<sub>2</sub> (data not shown), both the degradation of <sup>125</sup>I-U-T<sub>4</sub> and the formation of <sup>125</sup>I-DIT were significantly increased. Enrichment of homogenates with

GSH (5 mM) prevented these effects of endotoxin administration. The degradation of <sup>125</sup>I-DIT was not affected by endotoxin treatment. GSH decreased the degradation of <sup>125</sup>I-DIT slightly, but this effect was not consistently seen.

The effect of endotoxin treatment on T<sub>3</sub> neogenesis was assessed in separate experiments in which the treatment protocol was the same as that described above. In these experiments, however, 25% rather than 10% (wt/vol) liver homogenates were used and preparations were incubated with <sup>125</sup>I-β-T<sub>4</sub> under N<sub>2</sub> for 3 h. Like aminotriazole, endotoxin given in vivo decreased in vitro T<sub>3</sub> neogenesis. In specimens from control and endotoxin-treated animals, respectively, degradation of <sup>125</sup>I-β-T<sub>4</sub> was 31.6±0.3 vs. 26±1.5% (*P* < 0.05); formation of <sup>125</sup>I-T<sub>3</sub> was 8.4±0.3 vs. 5.4±0.9% (*P* < 0.05); and formation of <sup>125</sup>I-I<sup>-</sup> was 21.6±0.3 vs. 19.2±1.0% (NS).

**Lactoperoxidase-catalyzed formation of DIT (Fig. 5).** To determine which of the various components of the putative system that forms DIT from T<sub>4</sub> is required, a cell-free model of this reaction was studied. Preparations contained <sup>125</sup>I-U-T<sub>4</sub> as substrate, lactoperoxidase (40 μg), an H<sub>2</sub>O<sub>2</sub>-generating system (1 mg glucose and 0.7 U glucose oxidase) and catalase (6,000 units), alone or variously combined in 1 ml of buffer. Mixtures were incubated under O<sub>2</sub> for 1 h. Only slight formation of <sup>125</sup>I-DIT and degradation of <sup>125</sup>I-U-T<sub>4</sub> occurred in buffer alone, or in buffer containing only the H<sub>2</sub>O<sub>2</sub>-generating system, lactoperoxidase, or catalase. In the presence of lactoperoxidase and the H<sub>2</sub>O<sub>2</sub>-generating system, however, the degradation of the substrate and the formation of <sup>125</sup>I-labeled DIT and <sup>125</sup>I-I<sup>-</sup> were markedly stimulated. As expected, addition of catalase to this mixture markedly attenuated the metabolism of T<sub>4</sub> to DIT.

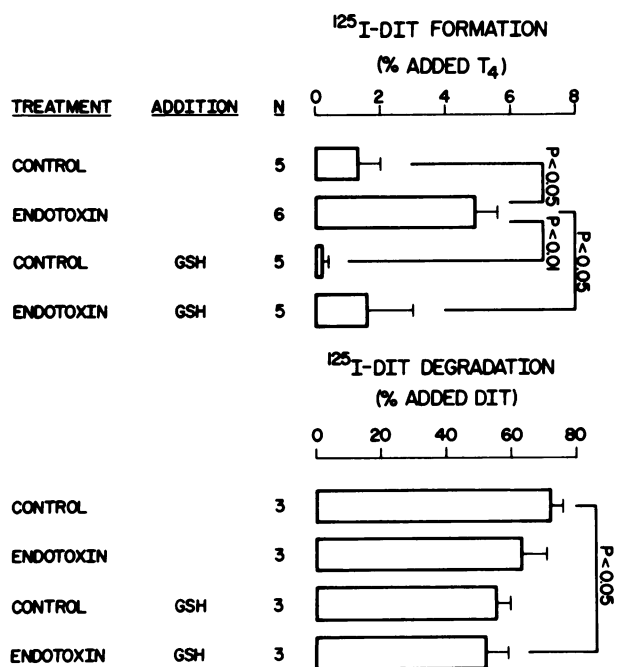


FIGURE 4 Aliquots of homogenates (1:9, wt/vol) of livers of control rats and those injected with endotoxin were incubated under the same conditions to assess <sup>125</sup>I-DIT formation from <sup>125</sup>I-U-T<sub>4</sub> (shown above) and <sup>125</sup>I-DIT degradation (shown below).

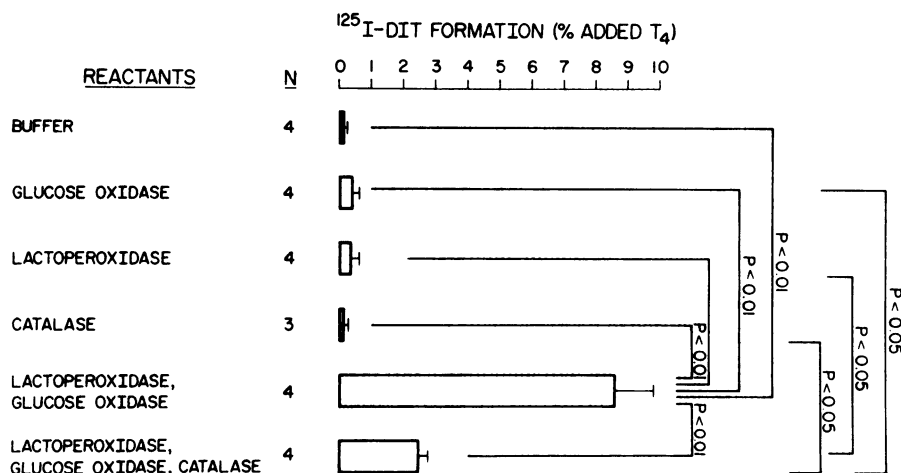


FIGURE 5 The formation of  $^{125}\text{I}$ -DIT from  $^{125}\text{I}$ -U- $\text{T}_4$  by lactoperoxidase was assessed in a cell-free system. Reaction mixtures were variously constituted with buffer; lactoperoxidase ( $40\text{ }\mu\text{g/ml}$ ); glucose ( $1\text{ mg/ml}$ ), glucose oxidase ( $0.7\text{ units/ml}$ ); and catalase ( $6,000\text{ units/ml}$ ). Mixtures were incubated with  $^{125}\text{I}$ -U- $\text{T}_4$  ( $0.25\text{ }\mu\text{Ci/ml}$ ,  $0.11\text{ ng/ml}$ ) in  $\text{O}_2$  for  $1\text{ h}$ .

## DISCUSSION

According to currently prevailing concepts,  $\text{T}_4$  is deiodinated by the enzymatic removal of a single iodine atom from either the 5'- or the 5-position of its molecule, yielding  $\text{T}_3$  or  $\text{rT}_3$ , respectively. Before the importance of these monodeiodinations was recognized, Galton and Ingbar (3, 4) reported that the deiodination of  $\text{T}_4$ , both in the intact rat and in preparations of rat tissues, was markedly enhanced by inhibitors of the enzyme catalase. On the basis of that and other evidence, they suggested that the deiodination of  $\text{T}_4$  might be mediated by tissue peroxidases.

The pathway involved could not be deduced, however, since the reaction, as monitored with  $\text{T}_4$  labeled in its outer ring ( $^{131}\text{I}$ - $\beta$ - $\text{T}_4$ ), yielded only  $^{131}\text{I}$ - $\text{I}^-$  and  $^{131}\text{I}$ -OM, neither iodothyronines nor other compounds labeled with radioiodine being detected.

Theoretically, it appeared possible that the peroxidase-mediated pathway of  $\text{T}_4$  metabolism might involve the generation of  $\text{T}_3$ , but that no  $\text{T}_3$  would appear to have been formed, owing to its rapid degradation under the conditions of study. In the present studies, to evaluate this possibility, the capacity of aminotriazole to accelerate the metabolism of the substrate  $\text{T}_4$  and its deiodination product,  $\text{T}_3$ , was assessed in rat liver homogenates, with either  $^{125}\text{I}$ - $\beta$ - $\text{T}_4$  or  $^{125}\text{I}$ - $\beta$ - $\text{T}_3$  as substrates. The results of these studies clearly showed that rapid degradation could not be responsible for the exceedingly small quantities of  $\text{T}_3$  seen in incubations with  $\text{T}_4$ . This indicated that the pathway of  $\text{T}_4$  metabolism stimulated by aminotriazole did not lead to the formation of  $\text{T}_3$ .

Accordingly, an alternative mechanism for this reaction was postulated; this involved scission of the ether bond to produce DIT from the inner portion of the molecule and an unstable, readily deiodinated, moiety from the outer ring. Indications that this might indeed be the case were provided by the results of earlier studies. Plaskett (15) and, later, Wynn and Gibbs (16) showed that the nature of radioiodine-labeled products of labeled  $\text{T}_4$  metabolism was influenced by the position of the radioiodine label with respect to the ether bond. Preparations of rat liver metabolized  $^{131}\text{I}$ - $\beta$ - $\text{T}_4$  to yield mainly  $^{131}\text{I}$ - $\text{I}^-$ , whereas they degraded  $\text{T}_4$  labeled with  $^{131}\text{I}$  in its inner ring mainly to form  $^{131}\text{I}$ -DIT and  $^{131}\text{I}$ -labeled protein that contained large proportions or covalently-bound  $^{131}\text{I}$ -DIT.<sup>3</sup>

It was clear that the direct demonstration of the formation of DIT from  $\text{T}_4$ , which could occur only if the ether link of the  $\text{T}_4$  molecule were cleaved, would require use of a labeled  $\text{T}_4$  that contained  $^{125}\text{I}$  in its inner ring. To meet this need, we obtained uniformly labeled  $\text{T}_4$  from iodine-deficient rats given  $^{125}\text{I}$ - $\text{I}^-$ . With  $^{125}\text{I}$ -U- $\text{T}_4$  as substrate, we were able to demonstrate conclusively the presence of an  $\text{O}_2$ -dependent pathway of DIT formation in homogenates of rat liver. Though negligibly active in control homogenates, the

<sup>3</sup> Although it is theoretically possible that this reaction led to the formation of  $\text{rT}_3$ , which was then completely deiodinated, this appears unlikely.  $\text{rT}_3$  metabolism yields  $3,3'$ - $\text{T}_2$  and iodide and does not lead to the formation of significant quantities of origin material, as was seen in the present studies.

pathway could be greatly activated by a variety of experimental manipulations.

There seems little reason to doubt that the product of  $^{125}\text{I}$ -U- $\text{T}_4$  metabolism that we identify as  $^{125}\text{I}$ -DIT was indeed that compound. First, in experiments to this point, good concordance was seen between measurements of the compound in question, which behaved like DIT in both paper and column chromatography. Second, net formation of the compound was increased by addition of the iodotyrosine dehalogenase inhibitor, DNT. Third, a compound of identical chromatographic properties was generated from  $^{125}\text{I}$ -U- $\text{T}_3$  and its net generation was also increased by DNT.

Several lines of evidence support the likelihood that this formation of DIT from  $\text{T}_4$  is mediated by tissue peroxidases. Among these is the finding that the reaction was activated by the catalase inhibitors aminotriazole and endotoxin under aerobic, but not anaerobic, conditions. Furthermore, this activation was markedly suppressed by substrates of peroxidase and by GSH, a reductant of  $\text{H}_2\text{O}_2$ . In addition, as might be expected, the reaction was stimulated by the sulfhydryl inhibitor NEM, but this too occurred only in an atmosphere of  $\text{O}_2$ . Lastly, in a cell-free system, the reaction was catalyzed by lactoperoxidase in the presence of an  $\text{H}_2\text{O}_2$ -generating system, was markedly inhibited by catalase, and no effect was seen with the  $\text{H}_2\text{O}_2$ -generating system alone.

Several of the factors that altered the formation of DIT from  $\text{T}_4$  also influenced  $\text{T}_3$  neogenesis, but in a reciprocal manner. Thus, both aerobiosis and NEM enhanced DIT formation and suppressed  $\text{T}_3$  neogenesis, whereas anaerobiosis and GSH had the converse effects. Furthermore, the catalase inhibitors aminotriazole and endotoxin were inhibitory to  $\text{T}_3$  neogenesis, but greatly increased DIT formation.

A hypothetical basis for the effects of the various experimental manipulations on the formation of DIT and  $\text{T}_3$  is suggested in Fig. 6. It appears that these manipulations ultimately may influence the levels of cofactors critical to these reactions, GSH for  $\text{T}_3$  neogenesis (17–20), and  $\text{H}_2\text{O}_2$  for DIT formation. The relative availability of these cofactors would be expected to determine which of these pathways would be activated and which would be suppressed. Moreover, the reciprocal influences of the experimental manipulations on these pathways is thought to derive from the interaction of the cofactors, whereby  $\text{H}_2\text{O}_2$  undergoes enzymatic reduction by GSH. Hence, according to this formulation, inhibition of catalase would favor DIT formation by permitting the accumulation of  $\text{H}_2\text{O}_2$ , thereby promoting peroxidase-mediated cleavage of the ether bond of  $\text{T}_4$ . Conversely, this would inhibit  $\text{T}_3$  neogenesis by leading to GSH depletion,  $\text{H}_2\text{O}_2$  ac-

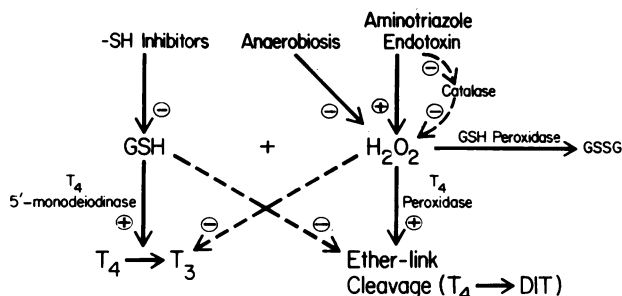


FIGURE 6 A hypothetical mechanism for the reciprocal regulation of pathways of  $\text{T}_4$  metabolism in rat liver that cleave the ether link to form DIT and that monodeiodinate the  $\text{T}_4$  molecule at the 5'-position to form  $\text{T}_3$ .

cumulation, or both. Depletion of hepatic GSH, as by NEM, would inhibit  $\text{T}_3$  neogenesis and promote DIT formation, presumably by increasing the availability of  $\text{H}_2\text{O}_2$  for peroxidations.

Until recently, there had been no direct demonstration that a DIT-forming pathway of  $\text{T}_4$  metabolism exists *in vivo*. Earlier studies seemed to indicate that the major pathways of  $\text{T}_4$  metabolism left the ether bond of the  $\text{T}_4$  molecule intact. Pittman et al. (21–23) showed that after the administration of  $\text{T}_4$  variously labeled with either  $^{14}\text{C}$  or  $^3\text{H}$  in its outer ring, inner ring, or alanine side chain, a major fraction of administered radioactivity was recovered in urinary metabolites containing radionuclides from the three portions of the  $\text{T}_4$  molecule. Although these studies clearly suggested that the major pathways of  $\text{T}_4$  metabolism do not involve the cleavage of the ether bond, they were not strictly quantitative and could not rigorously exclude, therefore, the possibility that some portion of  $\text{T}_4$  was metabolized by cleavage of the ether link to yield DIT.

Little can be concluded from an examination of the concentration of DIT in serum. As judged from highly refined radioimmunoassays (24), serum concentrations of DIT are extremely low (on the order of 7 ng/100 ml) and it cannot be judged with certainty whether the DIT arises from thyroid secretion or iodothyronine metabolism. Nonetheless, there is now compelling evidence, apart from that presented here, that pathways for the cleavage of the ether link of iodothyronines do exist or are, in fact, operative in the tissues of man and rat. Thus, formation of large proportions of  $^{125}\text{I}$ -DIT (~50%) from  $\text{T}_4$  labeled with  $^{125}\text{I}$  in its inner ring has recently been demonstrated to take place in phagocytosing human polymorphonuclear leukocytes (25). In accord with our suggestion that ether-link cleavage of  $\text{T}_4$  is mediated by  $\text{H}_2\text{O}_2$  and catalyzed by a peroxidase, formation of DIT by leukocytes was found to

be enzymic in nature; was inhibited by propylthiouracil, a peroxidase inhibitor; and was not carried out by leukocytes from patients with chronic granulomatous disease, a disorder in which phagocytosis is not accompanied by an oxidative burst.

Evidence of a similar process in rats is equally convincing. We have recently demonstrated by a double-isotope derivative technique that rats given the inhibitor of DIT deiodination 3-mononitrotyrosine (26), convert  $T_4$  in part to metabolites of DIT (27). Finally, administration of  $^{125}\text{I}$ -labeled 3,5-diiodothyronine to rats leads to the appearance of  $^{125}\text{I}$ -DIT in the serum (25).

If ether-link cleavage is an alternate pathway of  $T_4$  metabolism in man, at least under certain circumstances, this might help to explain certain aspects of peripheral  $T_4$  metabolism that are poorly understood at present. For example, under certain circumstances, the sum of the apparent production rates of  $T_3$  and  $rT_3$  is far less than the overall rate of deiodination of  $T_4$ . This is characteristically true in several clinical conditions that lead to impaired peripheral production of  $T_3$ , such as starvation (28, 29), and hepatic cirrhosis (30, 31). Here, the overall degradation of  $T_4$  is unchanged and  $T_3$  production is markedly reduced, while  $rT_3$  production rates are only slightly increased, findings that suggest that  $T_4$  metabolism has been diverted to other pathways. Additionally, in acute febrile illness, turnover of  $T_4$  can be greatly accelerated (32); and this too is probably not due to an increase in the generation of  $T_3$  or  $rT_3$ . Hence, it is conceivable that, as in normal rat liver so in normal man, the pathway for generating DIT from  $T_4$  is dormant, awaiting activation by factors that concomitantly inhibit monodeiodination of  $T_4$  to  $T_3$ .

## ACKNOWLEDGMENT

This work was supported in part by grant AM 18416 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health.

## REFERENCES

- Utiger, R. D. 1974. Serum triiodothyronine in man. *Annu. Rev. Med.* 25:289-302.
- Cavalieri, R. R., and B. Rapoport. 1977. Impaired peripheral conversion of thyroxine to triiodothyronine. *Annu. Rev. Med.* 28:57-65.
- Galton, V. A., and S. H. Ingbar. 1963. Role of peroxidase and catalase in the physiological deiodination of thyroxine. *Endocrinology*. 73:596-605.
- Galton, V. A., and S. H. Ingbar. 1964. Effect of catalase inhibitors on the deiodination of thyroxine. *Endocrinology*. 74:627-634.
- Balsam, A., and S. H. Ingbar. 1979. Ether-link cleavage: an alternate pathway of thyroxine metabolism. *Clin. Res.* 27:247A. (Abstr.)
- Inoue, K., and A. Taurog. 1967. Digestion of  $^{131}\text{I}$ -labeled thyroid tissue with maximum recovery of  $^{131}\text{I}$ -iodothyronines. *Endocrinology*. 81:319-332.
- Bellabarba, D., R. E. Peterson, and K. Sterling. 1968. An improved method for chromatography of iodothyronines. *J. Clin. Endocrinol. Metab.* 28:305-307.
- Sorimachi, K., and H. J. Cahnmann. 1977. A simple synthesis of [3,5- $^{125}\text{I}$ ]diiodo-L-thyronine and of [3,5- $^{125}\text{I}$ ]L-thyroxine of high specific activity. *Endocrinology*. 101:1276-1280.
- Sakurada, T., M. Rudolph, S. L. Fang, A. G. Vagenakis, L. E. Braverman, and S. H. Ingbar. 1977. Evidence that triiodothyronine and reverse triiodothyronine are sequentially deiodinated in man. *J. Clin. Endocrinol. Metab.* 46:916-922.
- Sorimachi, K., and N. Ui. 1975. Ion-exchange chromatographic analysis of iodothyronines. *Anal. Biochem.* 67:157-165.
- Balsam, A., F. C. Sexton, and S. H. Ingbar. 1979. The influence of fasting, diabetes, and several pharmacological agents on the pathways of thyroxine metabolism in the rat. *J. Clin. Invest.* 62:415-424.
- Dunnett, C. W. 1970. Multiple comparisons. In *Statistics in Endocrinology*. J. W. McArthur and T. Colton, editors. MIT Press, Cambridge, MA. 1:86-87.
- Deckel, L. A., editor. 1977. *Worthington Enzyme Manual*. Worthington Biochemical Corp., Freehold, NJ. 67.
- Flohe, L., W. A. Gunzler, and R. Ladenstein. 1976. Glutathione peroxidase. In *Glutathione: Metabolism and Function*. I. M. Arias and W. B. Jakoby, editors. 6:115-135. Raven Press, New York.
- Plaskett, L. G. 1961. Studies on the degradation of thyroid hormones in vitro with compounds labeled in either ring. *Biochem. J.* 78:652-657.
- Wynn, J., and R. Gibbs. 1962. Thyroxine degradation. II. Products of thyroxine degradation by rat liver microsomes. *J. Biol. Chem.* 237:3499-3505.
- Balsam, A., S. H. Ingbar, and F. C. Sexton. 1979. Observations on the factors that control the generation of triiodothyronine from thyroxine in rat liver and the nature of the defect induced by fasting. *J. Clin. Invest.* 63:1145-1156.
- Chopra, I. J. 1978. Sulfhydryl groups and the monodeiodination of thyroxine to triiodothyronine. *Science (Wash. DC)*. 199:904-906.
- Harris, A. R. C., S.-L. Fang, L. Hinerfeld, L. E. Braverman, and A. G. Vagenakis. The role of sulfhydryl groups on the impaired hepatic 3',3,5-triiodothyronine generation from thyroxine in hypothyroid, starved, fetal, and neonatal rodent. *J. Clin. Invest.* 63:516-524.
- Kaplan, M. M. 1979. Subcellular alterations causing reduced hepatic thyroxine 5'-monodeiodinase activity in fasted rats. *Endocrinology*. 104:58-64.
- Pittman, C. S., T. Maruyama, and J. B. Chambers, Jr. 1968. Metabolism of the diiodotyrosyl moiety of specifically labeled thyroxines. *Endocrinology*. 83:489-494.
- Pittman, C. S., and J. B. Chambers, Jr. 1969. Carbon structure of thyroxine metabolites in urine. *Endocrinology*. 84:705-710.
- Pittman, C. S., V. H. Read, J. B. Chambers, Jr., and H. Nakafuji. 1970. The integrity of the ether linkage during thyroxine metabolism in man. *J. Clin. Invest.* 49:373-380.
- Meinhold, H., A. Beckert, and K. W. Wenzel. 1981. Circulating diiodotyrosine: studies of its serum concen-

- tration, source and turnover using radioimmunoassay after immunoextraction. *J. Clin. Endocrinol. Metab.* 53:1171-1178.
25. Burger, A. G., D. Engler, U. Buerger, M. Weissel, G. Steiger, S. H. Ingbar, R. E. Rosin, and B. M. Babior. 1983. Ether link cleavage is the major pathway of iodothyronine metabolism in the phagocytosing human leukocyte and also occurs *in vivo* in the rat. *J. Clin. Invest.* 71:935-949.
  26. Green, W. L. 1968. Inhibition of thyroidal iodotyrosine deiodination by tyrosine analogues. *Endocrinology.* 83:336-347.
  27. Balsam, A., M. I. Surks, and S. H. Ingbar. 1980. Demonstration of *in vivo* ether-link cleavage of thyroxine to yield diiodotyrosine. *Clin. Res.* 28:255A. (Abstr.)
  28. Eisenstein, Z., S. Hagg, A. G. Vagenakis, S. L. Fang, B. Ransil, A. Burger, A. Balsam, L. E. Braverman, and S. H. Ingbar. 1978. Effect of starvation on the production and peripheral metabolism of 3,3',5'-triiodothyronine in euthyroid obese subjects. *J. Clin. Endocrinol. Metab.* 47:889-893.
  29. Suda, A. K., C. S. Pittman, T. Shimizu, and J. B. Chambers, Jr. 1978. The production and metabolism of 3,5,3'-triiodothyronine and 3,3',5'-triiodothyronine in normal and fasting subjects. *J. Clin. Endocrinol. Metab.* 47:1311-1319.
  30. Chopra, I. J., D. H. Solomon, U. Chopra, R. T. Young, and G. N. Chua Teco. 1974. Alterations in circulating thyroid hormones and thyrotropin in hepatic cirrhosis: evidence for euthyroidism despite subnormal serum triiodothyronine. *J. Clin. Endocrinol. Metab.* 39:501-511.
  31. Nomura, S., C. S. Pittman, J. B. Chambers, Jr., M. W. Buck, and T. Shimizu. 1975. Reduced peripheral conversion of thyroxine to triiodothyronine in patients with hepatic cirrhosis. *J. Clin. Invest.* 56:643-652.
  32. Gregerman, R. I., and N. Solomon. 1967. Acceleration of thyroxine and triiodothyronine turnover of during bacterial pulmonary infections and fever: implications for the functional state of the thyroid during stress and senescence. *J. Clin. Endocrinol. Metab.* 27:93-105.