Formation of Diiodotyrosine from Thyroxine

ETHER-LINK CLEAVAGE, AN ALTERNATE PATHWAY OF THYROXINE METABOLISM

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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}I either at the 5′-position of the outer-ring (^{125}I-β-T_4) or uniformly in both the outer and inner rings (^{125}I-U-T_4). In homogenates incubated with ^{125}I-β-T_4 in an atmosphere of O_2, the catalase inhibitor aminotriazole greatly enhanced T_4 degradation, promoting the formation of large proportions of ^{125}I-labeled iodide (^{125}I-I^-) and chromatographically immobile origin material (^{125}I-OM), but only a minute proportion of ^{125}I-labeled 3,5,3′-triiodothyronine (^{125}I-T_3) (T_3 neogenesis). In an atmosphere of N_2, in contrast, homogenates produced much larger proportions of ^{125}I-T_3, and aminotriazole had no effect. In incubations with ^{125}I-U-T_4, under aerobic conditions, control homogenates degraded T_4 slowly; formation of ^{125}I-labeled 3,5-diiodotyrosine (^{125}I-DIT) was seen only occasionally and in minute proportions. However, in homogenates incubated under O_2, but not N_2, aminotriazole consistently elicited the formation of large proportions of ^{125}I-DIT, indicating that the ether link of T_4 was being cleaved by an O_2-dependent process.

Formation of ^{125}I-DIT in the presence of aminotriazole and 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}I-DIT from ^{125}I-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}I-DIT as substrate revealed it to be rapidly deiodinated by liver homogenates under aerobic conditions. Recovery of ^{125}I-DIT from ^{125}I-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}I-U-T_4 as substrates, none of the factors that altered the proportion of ^{125}I-DIT found after incubations with ^{125}I-U-T_4 did so by altering the degradation of the ^{125}I-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 H_2O_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)[1] in man and animals is reductive mon-

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[1] *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-β-T_4, ^{125}I-β-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-β-T_4, sp act 50–70 Ci/g and ^{125}I-β-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 England 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 μl 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/μg)[2] 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/μg)[2] 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-β-T_4 (~1 μCi/ml, 0.020 μg/ml); ^{125}I-U-T_4 (0.25 μCi/ml), 0.11

diiodotyrosine; DNT, 3,5-dinitrotyrosine; HTA, hexane/tertiary amyl alcohol/2 N ammonia, 1:10:11; ^{125}I-β-T_3, outer-ring labeled ^{125}I-T_3; ^{125}I-β-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.

[2] 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}I-β-T_3 (~1.3 $\mu Ci/ml$, 0.025 $\mu g/ml$); ^{125}I-U-T_3 (0.25 $\mu Ci/ml$, 0.11 ng/ml); or DIT labeled with ^{125}I or ^{131}I (1.0 $\mu Ci/ml$). Reaction vessels were continuously flushed with purified O_2 or N_2 and were incubated in a metabolic incubator at 37°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°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}I-β-T_4 or ^{125}I-β-T_3 metabolism, and BAW for studies of ^{125}I-U-T_4, ^{125}I-U-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}I-β-T_4 (Table I A) and ^{125}I-β-T_3. As judged from analyses in the HTA and BAW systems, whether incubated under O_2 or N_2, control rat liver homogenates formed the following ^{125}I-labeled products from ^{125}I-β-T_4: T_3, I^-, and chromatographically immobile origin material (OM). Under O_2, enrichment with aminotriazole (60 mM) greatly enhanced both the degradation of ^{125}I-β-T_4 and the formation ^{125}I-I^- and ^{125}I-OM, but did not significantly affect the very slight net formation of ^{125}I-T_3 that occurred under these conditions. Under N_2, formation of T_3 was greatly increased, but aminotriazole was totally without effect. Formation of ^{125}I-I^- and ^{125}I-OM was comparable when HTA or BAW was used for analysis, and ^{125}I-DIT was not detected in either system.

Additional experiments were carried out to determine whether the very slight apparent formation of ^{125}I-T_3 from ^{125}I-T_4 in the presence of aminotriazole and O_2, despite the extensive deiodination of T_4 that occurred under these conditions, could be explained by a very rapid degradation of T_3. For this purpose, the metabolism of either ^{125}I-β-T_4 and of ^{125}I-β-T_3 by paired aliquots of liver homogenates was examined. In the presence of aminotriazole and O_2, the degradation of ^{125}I-β-T_4 increased from 5.5±0.8 (mean±SE) to 68.4±1.9% ($P < 0.001$), whereas the formation of ^{125}I-T_3 was again not significantly changed (1.7±0.2 vs. 1.6±0.1%). In vessels incubated in parallel, the degradation of ^{125}I-β-T_3 was increased by aminotriazole

TABLE I
The Effect of Aminotriazole on the Metabolism of ^{125}I-β-T_4 in Rat Liver Homogenates

Treatment	Incubation atmosphere	*n*	^{125}I-β-T_4 degradation*	^{125}I-T_3 formation	^{125}I-I^- formation	^{125}I-OM formation
				% added T_4		
A Aminotriazole in vitro‡						
Control	O_2	3	12.0±1.4	1.4±0.2	4.5±1.0§	5.2±0.1
Experimental	O_2	3	79.4±14.6§	2.1±0.4	57.4±12.7	18.8±2.8§
Control	N_2	3	22.7±0.8	7.0±0.2	13.6±0.6	0.7±0.01
Experimental	N_2	3	20.7±0.9	7.2±0.6	11.7±1.0	0.8±0.1
B Aminotriazole in vivo‖						
Control	O_2	4	15.0±0.9	4.1±0.9	3.5±0.5	5.4±0.3
Experimental	O_2	4	95.8±0.3¶	2.3±0.8	78.9±0.6¶	14.4±0.3¶
Control	N_2	7	14.0±2.7	4.6±0.4	5.5±2.4	3.7±1.0
Experimental	N_2	7	11.8±1.4	2.8±0.2¶	5.4±1.3	2.0±0.2

* Values shown are mean±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}I-β-T_4 (1 $\mu Ci/ml$, 0.020 $\mu g/ml$) and incubated in O_2 or 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}I-β-T_4 and were incubated in O_2 or N_2 for 3 h.

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

from 2.0±0.2 to 48.5±2.3% ($P < 0.001$). These data suggested that as much as half of the $^{125}I-T_3$ formed from $^{125}I-T_4$ in the presence of aminotriazole would have persisted. On this basis, it can be deduced that increased 5'-monodeiodination of T_4 to yield T_3 could have accounted for only a small fraction of the 12-fold increase in T_4 deiodination that aminotriazole induced.

Effect of aerobic preincubation with aminotriazole on T_3 neogenesis (Fig. 1). The foregoing experiments did not permit firm conclusions concerning the effect of aminotriazole on the generation of T_3 from T_4, since aminotriazole was inactive under the anaerobic conditions that were optimal for the formation of T_3. Hence, studies were designed to allow the effects of aminotriazole and other additives to take place during a period of preincubation in O_2, while the metabolism of $^{125}I-\beta-T_4$ was allowed to take place during a subsequent period of incubation in 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°C under O_2 for 5 min. $^{125}I-\beta-T_4$ was added to all specimens and they were then incubated under N_2 for 3 h. Compared with homogenates not preincubated, homogenates preincubated at 37°C without additives formed much less $^{125}I-T_3$. When the latter contained aminotriazole, further inhibition of $^{125}I-T_3$ formation was evident. Enrichment with GSH (5 mM) prevented the decrease in T_3 neogenesis caused by preincubation at 37°C, and attenuated the inhibitory effect of aminotriazole.

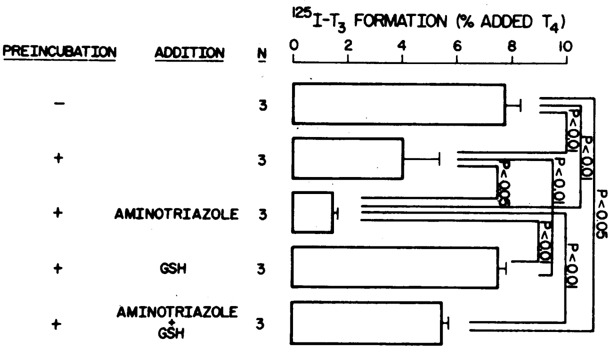


FIGURE 1 Homogenates of rat liver (1:3, wt/vol) were variously enriched with aminotriazole (60 mM) and GSH (5 mM) and preincubated at 37°C in O_2 for 5 min. Control specimens were kept at 0°C (not preincubated) during this time. $^{125}I-\beta-T_4$ (1 μCi/ml, 0.020 μg/ml), was then added and specimens were incubated under N_2 for 3 h to assess the formation of $^{125}I-T_3$. 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 $^{125}I-\beta-T_4$ (Table I B). For these experiments, animals were given two injections of aminotriazole (0.4 g/100 g body wt i.p.) 16 h apart and were killed 4 h after the second injection. Liver homogenates were prepared, enriched with $^{125}I-\beta-T_4$, and incubated under O_2 or N_2 during the ensuing 3 h. In specimens incubated under O_2, prior administration of aminotriazole greatly increased the degradation of $^{125}I-T_4$ and the generation of $^{125}I-I^-$ and ^{125}I-OM, and markedly decreased the percentage of $^{125}I-T_3$ generated from $^{125}I-T_4$. In contrast, when homogenates from rats treated with aminotriazole were incubated under N_2, degradation of $^{125}I-\beta-T_4$ and formation of $^{125}I-I^-$ and ^{125}I-OM were unchanged, whereas the formation of $^{125}I-T_3$ was markedly and significantly decreased.

Aminotriazole in vitro: effects on the metabolism of $^{125}I-U-T_4$ and $^{125}I-U-T_3$. In ensuing studies, to permit the detection of metabolites bearing ^{125}I derived from the inner ring of the T_4 molecule, $^{125}I-U-T_4$ was used as the substrate (Table II A). Rat liver homogenates, containing either no additive or aminotriazole (60 mM), were enriched further with $^{125}I-U-T_4$ and incubated under either O_2 or N_2 for 1 h. Under these conditions, labeled products of $^{125}I-U-T_4$ metabolism included those found when $^{125}I-\beta-T_4$ was the substrate (i.e., OM, I^-, and T_3); however, under certain circumstances ^{125}I-DIT, but not ^{125}I-MIT, was found. In control homogenates, whether incubated under O_2 or N_2, formation of DIT was absent or negligible. However, under O_2, addition of aminotriazole led to the formation of readily detectable quantities of ^{125}I-DIT (~4–5% of added $^{125}I-U-T_4$). No such effect of aminotriazole was seen under N_2. Results with respect to the degradation of $^{125}I-U-T_4$, as well as the generation of labeled I^- and OM, were similar to those seen when $^{125}I-\beta-T_4$ served as substrate. As monitored with the HTA system, $^{125}I-T_3$ was routinely formed from $^{125}I-U-T_4$, but its formation was not regularly measured, since the effects of various experimental manipulations on T_3 neogenesis had been evaluated with $^{125}I-\beta-T_4$ as substrate.

Formation of ^{125}I-DIT from $^{125}I-U-T_4$ 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 $^{125}I-U-T_4$ under O_2 or N_2 for 1 h. Reaction mixtures were then extracted with 5 vol of ethanol; the extracts were evaporated completely under N_2, and their contents were dissolved in buffer. Before chromatography, specimens were further enriched with an extract of a Pronase hydrolysate of ^{131}I-labeled thyroglobulin to provide

TABLE II

The Effect of Aminotriazole on the Metabolism of ^{125}I-U-T_4 by Rat Liver Homogenates

Treatment	Incubation atmosphere	n	^{125}I-U-T_4 degradation*	^{125}I-DIT formation	^{125}I-I^- formation	^{125}I-OM formation
					% added T_4	
A Aminotriazole in vitro‡						
Control	O_2	7	2.7±1.1	0.2±0	0.9±0.4	1.3±0.6
Experimental	O_2	7	64.1±4.1§	4.1±0.7§	41.5±5.4§	15.0±4.0‖
Control	N_2	3	6.4±3.3	0.2±0.1	3.0±0.7	2.5±3.5
Experimental	N_2	3	2.4±1.3	0.2±0.2	2.1±1.1	0.2±0.2
B Aminotriazole in vivo¶						
Control	O_2	4	4.1±0.6	0.5±0.2	2.1±0.6	1.5±0.1
Experimental	O_2	4	74.0±1.0§	3.2±0.7‖	35.6±0.8§	29.8±0.6§
Control	N_2	4	14.2±2.2	1.4±0.2	13.0±0.7	1.2±0.5
Experimental	N_2	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}I-U-T_4 (0.25 μCi/ml, 0.11 ng/ml) and were incubated in O_2 or N_2 for 1 h.

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

‖ $P < 0.01$ vs. corresponding control group.

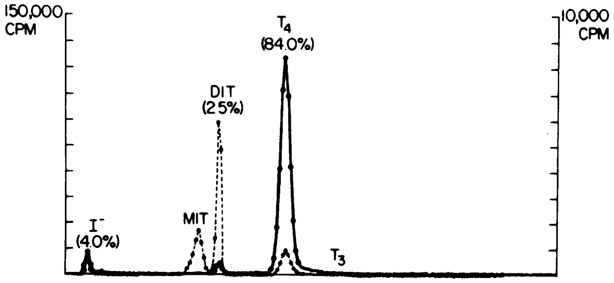
¶ Aminotriazole was administered to rats in two doses (0.4 g/100 g body wt i.p.) given 16 h apart. Homogenates (1:9, wt/vol) of liver were prepared 4 h after the second injection, enriched with ^{125}I-U-T_4, and incubated in O_2 or N_2 for 3 h.

^{131}I-DIT as a marker for any ^{125}I-DIT formed from ^{125}I-U-T_4. The results of one of three closely agreeing experiments are depicted in Fig. 2. Contamination of the substrate by ^{125}I-DIT was ~2% (upper panel). In homogenates that contained no aminotriazole, whether incubated under O_2 or N_2, and in those enriched with aminotriazole but incubated under N_2, the degradation of ^{125}I-U-T_4 was slight, and the percentage of ^{125}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}I-U-T_4 and increased proportions of ^{125}I-DIT and ^{125}I-I^- 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 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}I-U-T_4 or ^{125}I-DIT, to permit an evaluation of both DIT formation and degradation. In the absence of aminotriazole, DNT (10 mM) increased the apparent ^{125}I-DIT formation from 0.8±0.5 to 1.9±.1% (mean±SE). In the presence of the catalase inhibitor, DNT (10 mM) increased ^{125}I-DIT formation from 3.7±0.5 to 5.7±0.7%.

^{125}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}I-DIT added) and markedly, though less completely, in specimens enriched with aminotriazole (13.4±3.9% ^{125}I-DIT added). These findings indicate that, as expected, inhibition of DIT metabolism within liver homogenates leads to increased accumulation of ^{125}I-DIT from ^{125}I-U-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}I-U-T_3 were comparable to those seen when ^{125}I-U-T_4 was substrate. In the presence of O_2, aminotriazole greatly enhanced the degradation of ^{125}I-U-T_3 (4.6±2.3 vs. 52.7±23%, $P < 0.01$), and increased the formation of ^{125}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}I-U-T_4 (Table II B). In these experiments, performed according to the protocol described above for studies with ^{125}I-β-T_4, liver homogenates from either control or aminotriazole-treated rats were incubated with ^{125}I-U-T_4 under O_2 or N_2 for 3 h. Under O_2, homogenates from animals given aminotriazole displayed marked increases in both the degradation of



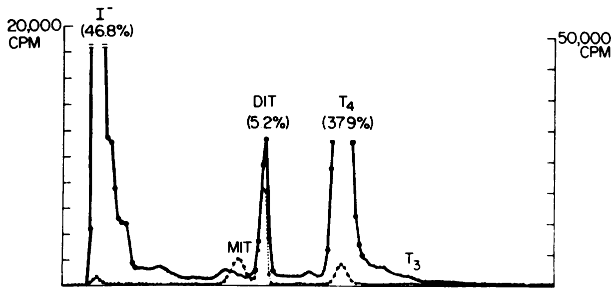


FIGURE 2 Homogenate of rat liver (1:9, wt/vol) was incubated with ^{125}I-U-T_4 (0.25 μCi/ml, 0.11 ng/ml) and aminotriazole (60 mM) in O_2 for 1 h and the reaction mixture was then extracted with 5 vol of ethanol. The nature of the extractable ^{125}I was determined by cation-exchange column chromatography, with a pronase hydrolysate of ^{131}I-thyroglobulin as a source of marker iodoamino acids. The scale for ^{125}I-radioactivity is shown on the left and that for ^{131}I-radioactivity is shown on the right. Representative chromatograms of the initial uniformly labeled ^{125}I-U-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}I-I^-; dotted lines, ^{131}I-labeled marker.

^{125}I-U-T_4 and the formation of ^{125}I-I^- and ^{125}I-OM. As in the in vitro studies, administration of aminotriazole led to the formation of increased quantities of ^{125}I-DIT. These effects of aminotriazole were not seen when specimens were incubated under N_2.

Effects of peroxidase substrates (Table III). To evaluate the possibility that the formation of DIT from 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}I-U-T_4 or ^{125}I-DIT under O_2 for 1 h. In these experiments too, aminotriazole produced marked stimulation of both the metabolism of ^{125}I-U-T_4 and the formation therefrom of ^{125}I-DIT. Neither AAP (1 mM) nor guaiacol (1 mM) had an effect on 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 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 T_4 was sought in experiments that tested the effects of adding GSH (5 mM), a cofactor for the reduction of H_2O_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}I-U-T_4 or ^{131}I-DIT under O_2 for 1 h. As was evident in earlier experiments, aminotriazole activated degradation of ^{125}I-U-T_4, enhancing the formation of ^{125}I-DIT, ^{125}I-I^-, and ^{125}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}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 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}I-U-T_4 or ^{125}I-DIT in homogenates incubated under O_2 for 3 h (Fig. 3). As expected, addition of NEM (10 mM) produced a marked stimulation of the degradation of ^{125}I-U-T_4, accompanied by a corresponding increase in the formation of ^{125}I-DIT, ^{125}I-I^-, and ^{125}I-OM, without affecting the degradation of ^{125}I-DIT. These effects of NEM were not seen in incubations carried out under N_2.

The effect of NEM on T_3 neogenesis was assessed in liver homogenates from four rats incubated with ^{125}I-β-T_4 under N_2 for 3 h. Under these conditions, enrichment with NEM (10 mM) decreased the degradation of ^{125}I-β-T_4 from 17.0±1.7 to 12.0±0.7% ($P < 0.05$), ^{125}I-T_3 formation from 5.6±0.5 to 2.6±0.4% ($P < 0.005$), and ^{125}I-I^- formation from 10.2±1.2 to 3.9±0.5% ($P < 0.005$).

Effects of endotoxin (Fig. 4). Overall deiodination of 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}I-U-T_4 in Rat Liver Homogenates

Experimental group*	Addition*	n‡	^{125}I-U-T_4 degradation	^{125}I-DIT formation	^{125}I-I^- formation‡	^{125}I-OM formation	^{131}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‖			$P < 0.05$ a vs. f c vs. f e vs. f $P < 0.01$ a vs. b b vs. c b vs. d b vs. e b vs. f	$P < 0.05$ d vs. f $P < 0.01$ a vs. b a vs. d b vs. c b vs. d b vs. e b vs. f c vs. d d vs. e	$P < 0.01$ a vs. b b vs. c b vs. d b vs. e b vs. f	$P < 0.05$ d vs. f e vs. f $P < 0.01$ a vs. b a vs. f b vs. c b vs. d b vs. e b vs. f c vs. f	$P < 0.05$ b 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}I-U-T_4 (0.25 μCi/ml, 0.11 ng/ml) or ^{131}I-DIT (1 μCi/ml) and incubated in O_2 for 1 h.

‡ n represents number of separate homogenates in which the metabolism of ^{125}I-U-T_4 was studied. For experiments with ^{131}I-DIT, n = 4.

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

‖ 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}I-U-T_4 in Rat Liver Homogenates

Experimental group*	Addition*	^{125}I-U-T_4 degradation	^{125}I-DIT formation	^{125}I-I^- formation	^{125}I-OM formation	^{131}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}I-U-T_4 (0.25 μCi/ml, 0.11 ng/ml) or ^{125}I-DIT (1 μCi/ml) and then incubated in O_2 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.

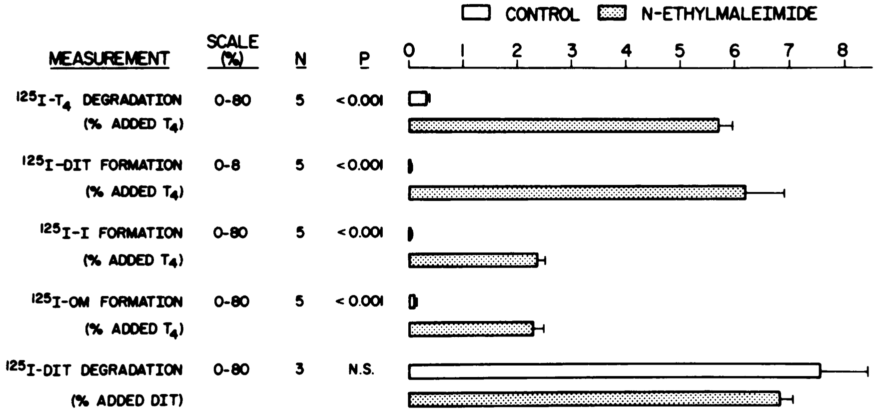


FIGURE 3 Homogenates of rat liver (1:9, wt/vol) were variously enriched with NEM (10 mM), ^{125}I-U-T_4 (0.25 μCi/ml, 0.11 ng/ml) or ^{125}I-DIT (1 μCi/ml) and incubated in O_2 for 1 h.

g body wt, 4 h before they were killed. Homogenates were incubated with ^{125}I-U-T_4 or ^{125}I-DIT under O_2 or N_2 for 3 h. In specimens from endotoxin-treated rats incubated aerobically, but not in those incubated under N_2 (data not shown), both the degradation of ^{125}I-U-T_4 and the formation of ^{125}I-DIT were significantly increased. Enrichment of homogenates with

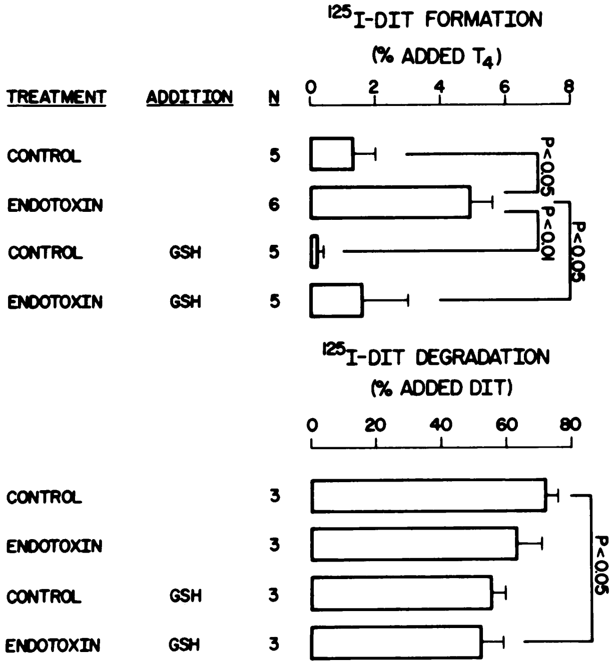


FIGURE 4 Aliquots of homogenates (1:9, wt/vol) of livers of control rats and those injected with endotoxin were incubated under the same conditions to assess ^{125}I-DIT formation from ^{125}I-U-T_4 (shown above) and ^{125}I-DIT degradation (shown below).

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

The effect of endotoxin treatment on T_3 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 ^{125}I-β-T_4 under N_2 for 3 h. Like aminotriazole, endotoxin given in vivo decreased in vitro T_3 neogenesis. In specimens from control and endotoxin-treated animals, respectively, degradation of ^{125}I-β-T_4 was 31.6±0.3 vs. 26±1.5% ($P < 0.05$); formation of ^{125}I-T_3 was 8.4±0.3 vs. 5.4±0.9% ($P < 0.05$); and formation of ^{125}I-I^- 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_4 is required, a cell-free model of this reaction was studied. Preparations contained ^{125}I-U-T_4 as substrate, lactoperoxidase (40 μg), an H_2O_2-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_2 for 1 h. Only slight formation of ^{125}I-DIT and degradation of ^{125}I-U-T_4 occurred in buffer alone, or in buffer containing only the H_2O_2-generating system, lactoperoxidase, or catalase. In the presence of lactoperoxidase and the H_2O_2-generating system, however, the degradation of the substrate and the formation of ^{125}I-labeled DIT and ^{125}I-I^- were markedly stimulated. As expected, addition of catalase to this mixture markedly attenuated the metabolism of T_4 to DIT.

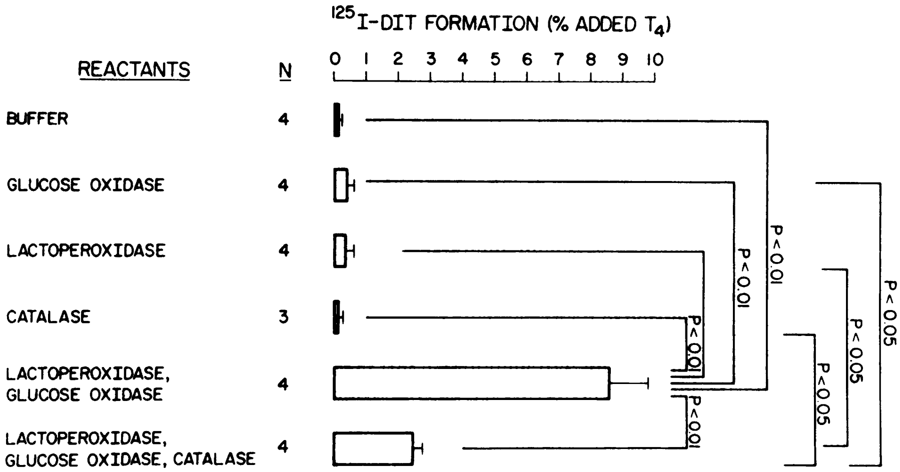


FIGURE 5 The formation of ^{125}I-DIT from ^{125}I-U-T_4 by lactoperoxidase was assessed in a cell-free system. Reaction mixtures were variously constituted with buffer; lactoperoxidase (40 μg/ml); glucose (1 mg/ml), glucose oxidase (0.7 units/ml); and catalase (6,000 units/ml). Mixtures were incubated with ^{125}I-U-T_4 (0.25 μCi/ml, 0.11 ng/ml) in O_2 for 1 h.

DISCUSSION

According to currently prevailing concepts, 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 T_3 or rT_3, respectively. Before the importance of these monodeiodinations was recognized, Galton and Ingbar (3, 4) reported that the deiodination of 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 T_4 might be mediated by tissue peroxidases.

The pathway involved could not be deduced, however, since the reaction, as monitored with T_4 labeled in its outer ring (^{131}I-β-T_4), yielded only $^{131}I^-$ and ^{131}I-OM, neither iodothyronines nor other compounds labeled with radioiodine being detected.

Theoretically, it appeared possible that the peroxidase-mediated pathway of T_4 metabolism might involve the generation of T_3, but that no 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 T_4 and its deiodination product, T_3, was assessed in rat liver homogenates, with either ^{125}I-β-T_4 or ^{125}I-β-T_3 as substrates. The results of these studies clearly showed that rapid degradation could not be responsible for the exceedingly small quantities of T_3 seen in incubations with T_4. This indicated that the pathway of T_4 metabolism stimulated by aminotriazole did not lead to the formation of 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 T_4 metabolism was influenced by the position of the radioiodine label with respect to the ether bond. Preparations of rat liver metabolized ^{131}I-β-T_4 to yield mainly $^{131}I^-$, whereas they degraded T_4 labeled with ^{131}I in its inner ring mainly to form ^{131}I-DIT and ^{131}I-labeled protein that contained large proportions or covalently-bound ^{131}I-DIT.[3]

It was clear that the direct demonstration of the formation of DIT from T_4, which could occur only if the ether link of the T_4 molecule were cleaved, would require use of a labeled T_4 that contained ^{125}I in its inner ring. To meet this need, we obtained uniformly labeled T_4 from iodine-deficient rats given ^{125}I-I^-. With ^{125}I-U-T_4 as substrate, we were able to demonstrate conclusively the presence of an O_2-dependent pathway of DIT formation in homogenates of rat liver. Though negligibly active in control homogenates, the

[3] Although it is theoretically possible that this reaction led to the formation of rT_3, which was then completely deiodinated, this appears unlikely. rT_3 metabolism yields 3,3′-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}I-U-T_4 metabolism that we identify as ^{125}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}I-U-T_3 and its net generation was also increased by DNT.

Several lines of evidence support the likelihood that this formation of DIT from 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 H_2O_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 O_2. Lastly, in a cell-free system, the reaction was catalyzed by lactoperoxidase in the presence of an H_2O_2-generating system, was markedly inhibited by catalase, and no effect was seen with the H_2O_2-generating system alone.

Several of the factors that altered the formation of DIT from T_4 also influenced T_3 neogenesis, but in a reciprocal manner. Thus, both aerobiosis and NEM enhanced DIT formation and suppressed T_3 neogenesis, whereas anaerobiosis and GSH had the converse effects. Furthermore, the catalase inhibitors aminotriazole and endotoxin were inhibitory to 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 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 T_3 neogenesis (17–20), and H_2O_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 H_2O_2 undergoes enzymatic reduction by GSH. Hence, according to this formulation, inhibition of catalase would favor DIT formation by permitting the accumulation of H_2O_2, thereby promoting peroxidase-mediated cleavage of the ether bond of T_4. Conversely, this would inhibit T_3 neogenesis by leading to GSH depletion, H_2O_2 accumulation, or both. Depletion of hepatic GSH, as by NEM, would inhibit T_3 neogenesis and promote DIT formation, presumably by increasing the availability of H_2O_2 for peroxidations.

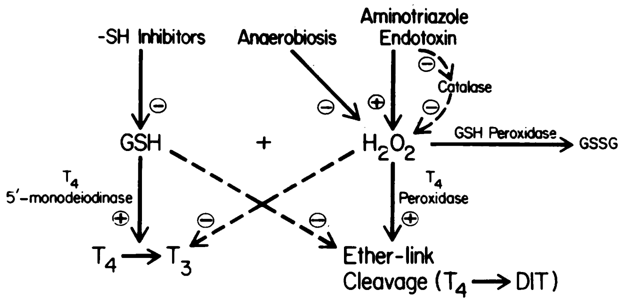


FIGURE 6 A hypothetical mechanism for the reciprocal regulation of pathways of T_4 metabolism in rat liver that cleave the ether link to form DIT and that monodeiodinate the T_4 molecule at the 5′-position to form T_3.

Until recently, there had been no direct demonstration that a DIT-forming pathway of T_4 metabolism exists in vivo. Earlier studies seemed to indicate that the major pathways of T_4 metabolism left the ether bond of the T_4 molecule intact. Pittman et al. (21–23) showed that after the administration of T_4 variously labeled with either ^{14}C or ^{3}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 T_4 molecule. Although these studies clearly suggested that the major pathways of 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 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}I-DIT (~50%) from T_4 labeled with ^{125}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 T_4 is mediated by H_2O_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}I-labeled 3,5-diiodothyronine to rats leads to the appearance of ^{125}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

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