Properties of a Rat Tissue Iodothyronine Deiodinase and Its Natural Inhibitor*

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ABSTRACT: B-ring deiodination of L-thyroxine (T_4) and L-triiodothyronine (T_3) by rat liver is enzymatic in nature, but the deiodinase activity in crude tissue homogenates is largely blocked by a naturally occurring inhibitor. The inhibitor is responsible for the time lag observed before deiodination takes place, and it is also responsible for the relative heat stability, pH dependence, and substrate inhibition of the system. All of these effects disappeared after the inhibitor was removed by dialysis. The specific activity of deiodinase was highest in liver, kidney, and heart microsomes, and the concentration of deiodinase remained the same in tissues of hypo-, eu-, and hyperthyroid animals. However, tissue levels of the inhibitor were highest in hypothyroid and lowest in hyperthyroid animals, suggesting that the natural inhibitor may play some role in metabolic control. The natural inhibitor was found in the soluble portion of the cell and was readily dialyzable. The identity of the inhibitor remains unknown, but a number of reducing substances such as ascorbate, reduced nicotinamide–adenine dinucleotide, reduced nicotinamide–adenine dinucleotide phosphate, cysteine, and reduced glutathione markedly inhibited deiodination. Therefore, the inhibitor may be a reducing substance that keeps either the active site on the enzyme or the substrate in the reduced form so that deiodination cannot proceed. Such a concept presupposes that deiodination is intimately concerned with oxidation of the thyronine ring structure, probably through the formation of a quinone. Removal of B-ring iodines may actually be secondary to oxidation of the phenol group, and the natural inhibitor may interfere with this oxidative step.

The earliest report of an *in vitro* system for the enzymatic deiodination of L-thyroxine (T_4) and L-triiodothyronine (T_3) was published in 1955 by Sprott and Maclagan. Since then, various aspects of the reaction have been studied, but the published results and conclusions are confusing. An inhibitor that prevents the deiodination reaction from following established enzyme kinetic theory is probably the major cause of this confusion (Stanbury *et al.*, 1960; Wynn and Gibbs, 1962; Nakagawa and Ruegamer, 1964; Yamamoto, 1964). However, a wide variation in substrate concentrations, pH optima, enzyme sources, cofactors, and assay techniques were used, and both enzymatic and nonenzymatic deiodination were observed. It has been suggested that some of the deiodination systems described may be artifactual (Jolin-Buzo *et al.*, 1966). In view of the current lack of clarity as to the mechanism and enzymatic nature of the iodothyronine deiodination reaction, a detailed and systematic study of the reaction was made, and the results are summarized below.

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Experimental Procedure

Animals and Tissue Homogenates. Holtzman strain male rats weighing 100–110 g were maintained on a Purina chow diet for at least 3 weeks prior to the experiment. The animals were sacrificed by decapitation, and 2.00-g samples of tissue were quickly removed and homogenized in a sufficient volume of buffer at 2° to yield a final volume of 20 ml. Phosphate (0.1 M), Krebs–Ringer phosphate, citrate, acetate, and Tris-maleate buffer systems were employed.

Substrates. All ^{131}I-labeled compounds (randomly labeled in the 3′ and 5′ positions of ring B) were obtained as carrier-free preparations (35 mc/mg), and were stored at −20°. Approximately 40 μc of ^{131}I-labeled substrate and 20 μmoles of unlabeled substrate were dissolved in distilled water adjusted to a final pH of 10 and a final volume of 25 ml. The substrate concentration in this stock solution was 800 mμmoles/ml.

Incubation and Assay Procedure. Homogenate (4 ml, 400 mg of fresh tissue) and buffer (8 ml) were pipetted into 25-ml erlenmeyer flasks. ^{131}I-Labeled substrate (200 μl, 160 mμmoles) was added, and the total volume was made up to 12.4 ml with buffer. The flasks were incubated in a Dubnoff shaker set at 120 oscillations/min and at a constant temperature of 37°. The incubations were always carried out in the dark in order to avoid light-induced deiodination. During the course of incubation, 3-ml aliquots of the incubation mixture were withdrawn at appropri-

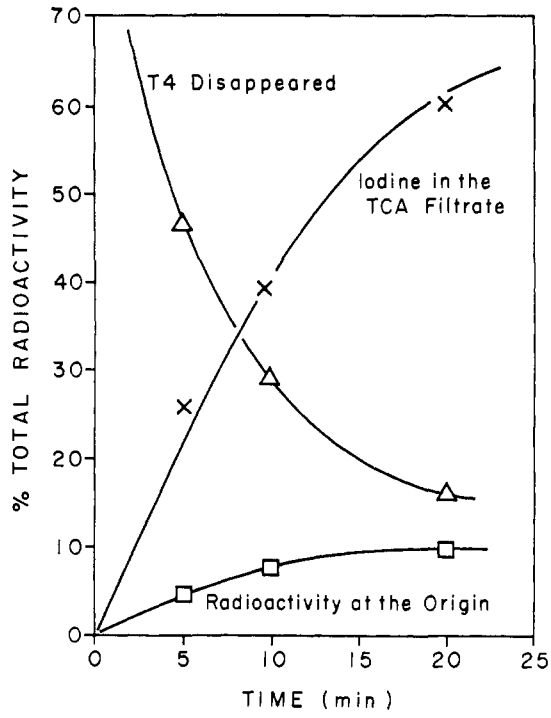

FIGURE 1: Relative amounts of radioactivity in the three major spots (origin material, iodide, and unreacted substrate) appearing on paper chromatograms of the incubation medium. Labeled T_4 (160 mμmoles) was incubated with 400 mg of liver homogenate at 37° and pH 5.8, and samples of the incubation mixture with added plasma were chromatographed with the butanol–dioxane–ammonia system previously described.

ate time intervals and mixed with 2 ml of human plasma. TCA[1] (3 ml of 20%) and 4 ml of 0.2% sodium iodide were added, and the sample was mixed in a Vortex mixer. The samples were centrifuged at 2000 rpm for 10 min, and the supernatants were recentrifuged. At least three aliquots of the incubation media were removed at different time intervals and processed in this manner so that the initial velocity of the reaction could be determined.

Two controls were always run along with the tissue samples. In one control, the amount of nonenzymatic deiodination was determined by incubating the substrate in the absence of enzyme but in the presence of all other substances. The other control was prepared by mixing all of the reactants together and immediately withdrawing an aliquot before the start of the incubation procedure. This blank was used to correct all incubated samples for the small amount of [^{131}I]iodide that is found in commercial preparations of ^{131}I-labeled substrates.

Counting Technique and Computations. Aliquots of 3 ml of the TCA supernatants (containing the [^{131}I]iodide liberated in the deiodination reaction) were counted in a γ-sensitive crystal scintillation well counter. Since the amounts of radioactive iodide found in the two controls described above were always the same, no detectable nonenzymatic deiodination took place and the average of the two [^{131}I]iodide values was used to correct for nonenzymatic deiodination.

The amount of substrate deiodinated was calculated as % substrate deiodinated = $100(I_e - I_c)/(I_t - I_c)$, where I_e is the amount of [^{131}I]iodide found in the sample supernatant, I_t is the total ^{131}I activity added to each flask as labeled substrate, and I_c is the average amount of [^{131}I]iodide found in the two control supernatants. Deiodinase activity was expressed as an initial reaction velocity, equal to the per cent of the labeled substrate deiodinated in 10 min by the equivalent of 400 mg of fresh tissue. The TCA precipitates were also counted to make certain that radioactivity was not lost during the experiment.

Paper Chromatographic Techniques. One-dimensional ascending paper chromatograms of plasma–incubation media and TCA supernatants of the media were made on Whatman paper No. 3MM to identify the labeled products of the deiodination reaction. Nonradioactive sodium iodide (1 mg) and the appropriate nonradioactive iodophenol (0.5 mg) were added/ml of plasma sample in order to ensure better resolution of the individual spots. The solvent systems used were: (1) 1-butanol–glacial acetic acid–water (75:10:15), (2) collidine–water–ammonia (125:44, v/v, and ammonium hydroxide atmosphere), and (3) 1-butanol–dioxane–ammonia (2 N) (4:1:5). Following development for 16–24 hr, the chromatograms were dried and cut into 0.5-cm strips. The amount of radioactivity on each strip was measured and R_F values were calculated for each radioactive peak.

Dialysis Technique. Various tissue preparations were dialyzed at 3–4° against phosphate buffer (pH 5.8) or glass-distilled water. Homogenates or cell fractions were placed in cellophane bags (0.75 in. in diameter), and the bags were placed in a motor-driven dialyzer (Oxford multiple dialyzer, Model B). The dialyzer had a capacity of 5 l., and the dialyzing medium was changed once during each 24-hr period.

Cell Fraction Studies. Tissue (2 g) was homogenized in 20 ml of 0.25 M sucrose solution at 2°, and various cell fractions were isolated by differential centrifugation (Hogeboom, 1955). The crude homogenate was centrifuged at 600*g* for 10 min. Supernatant 1 was carefully removed, and precipitate 1 was resuspended in phosphate buffer (pH 5.8). Supernatant 1 was centrifuged at 12,000*g* for 15 min, the resulting supernatant (2) was carefully removed, and the mitochondrial fraction was resuspended in phosphate buffer. The supernatant fraction left after the removal of all of the particulate fractions was termed the cell sap. The protein content of each cell fraction was deter-

[1] Abbreviations used: TCA, trichloroacetic acid; MIT and DIT, monoiodo- and diiodotyrosines; NADH, reduced nicotinamide–adenine dinucleotide; NADPH, reduced nicotinamide–adenine dinucleotide phosphate; Tu, thiouracil; FMN, flavin mononucleotide; FAD, flavin–adenine dinucleotide.

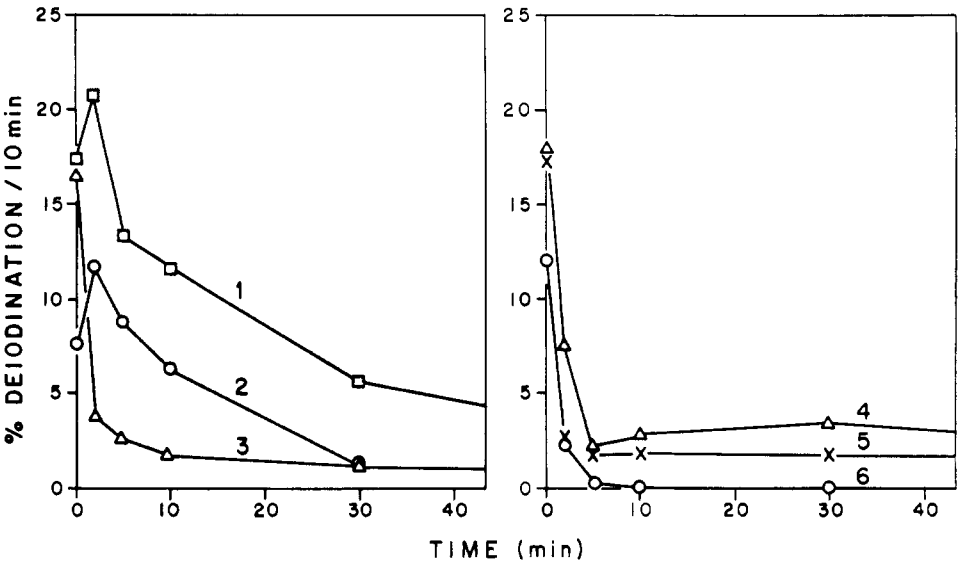

FIGURE 2: Relative stability of a rat liver T_4 deiodinase preparation subjected to heat treatment in a boiling water bath for varying periods of time. The liver enzyme preparations (pH 5.8) summarized below were heated in a boiling water bath for the various time intervals shown in the graphs. Following this treatment, deiodination velocities were determined for each of the heated preparations by adding 160 mμmoles of labeled T_4 to the equivalent of 400 mg of fresh liver and determining the rate of deiodination in the usual manner. Curve 1: undialyzed liver homogenate + 1.7×10^{-3} M Fe^{2+}. Curve 2: undialyzed liver homogenate. Curve 3: undialyzed liver microsomes plus 1.7×10^{-3} M Fe^{2+}. Curve 4: dialyzed liver homogenate plus 1.7×10^{-3} M Fe^{2+}. Curve 5: dialyzed liver homogenate. Curve 6: undialyzed liver microsomes.

mined by the Biuret procedure, and the specific deiodinase activity of each cell fraction was calculated as the per cent of the substrate deiodinated/10 min per mg of protein.

Results

Assay Procedure and Identification of Labeled Products. The deiodination reaction was always stopped by the addition of plasma, and the [^{131}I]iodide released from the substrate was separated from the unreacted substrate by TCA precipitation of the protein fraction. Provided the liberated iodide does not iodinate other proteins and that it is the only labeled substance present in the TCA supernatant, it should be possible to determine the amount of [^{131}I]iodide released by simply counting the TCA supernatants. To test the first of these assumptions, 160 mμmoles of sodium iodide containing approximately 60,000 cpm of carrier-free [^{131}I]iodide was incubated for 60 min at 37° with 400 mg of fresh liver homogenate in a total volume of 12.4 ml of pH 5.8 phosphate buffer. Incubations were performed with and without the addition of 1.7×10^{-3} M ferrous ions, since this concentration of ferrous ions increases the rate of deiodination under some conditions (Nakagawa and Ruegamer, 1964). Plasma and TCA were added and the resulting TCA precipitate was washed with 0.2% sodium iodide solution and centrifuged. More than 95% of the initial radioactivity was always found in the TCA supernatant, indicating that protein iodination did not take place either in the presence or absence of ferrous ions.

In order to test the second assumption that [^{131}I]-iodide is the sole radioactive product of ^{131}I-labeled T_4 and T_3 deiodination, samples of both the reaction mixture with added plasma and the TCA supernatants prepared from this mixture were chromatographed on paper. The only radioactive spots observed with samples of the plasma–incubation medium were unreacted T_4 and T_3, iodide, and origin material.[2] Radioactive iodide was the only spot found on chromatograms of the TCA filtrates prepared from the incubation media. Neither the addition of ferrous ions to the incubation mixture nor dialysis of the crude liver homogenate before incubation affected the radioactive products of the reaction. The relationship between the amounts of unreacted T_4, iodide, and origin material present with respect to time are shown in Figure 1. Since radioactive iodide was the only radioactive product found in TCA supernatants of the incubation medium, and since proteins were not iodinated under these conditions, TCA supernatant radioactivities were used to calculate initial reaction velocities in all subsequent experiments.

Oxygen Requirement. When incubation was carried

[2] Origin material is the the term frequently used to denote all radioactive substances that remain at the origin on paper chromatograms.

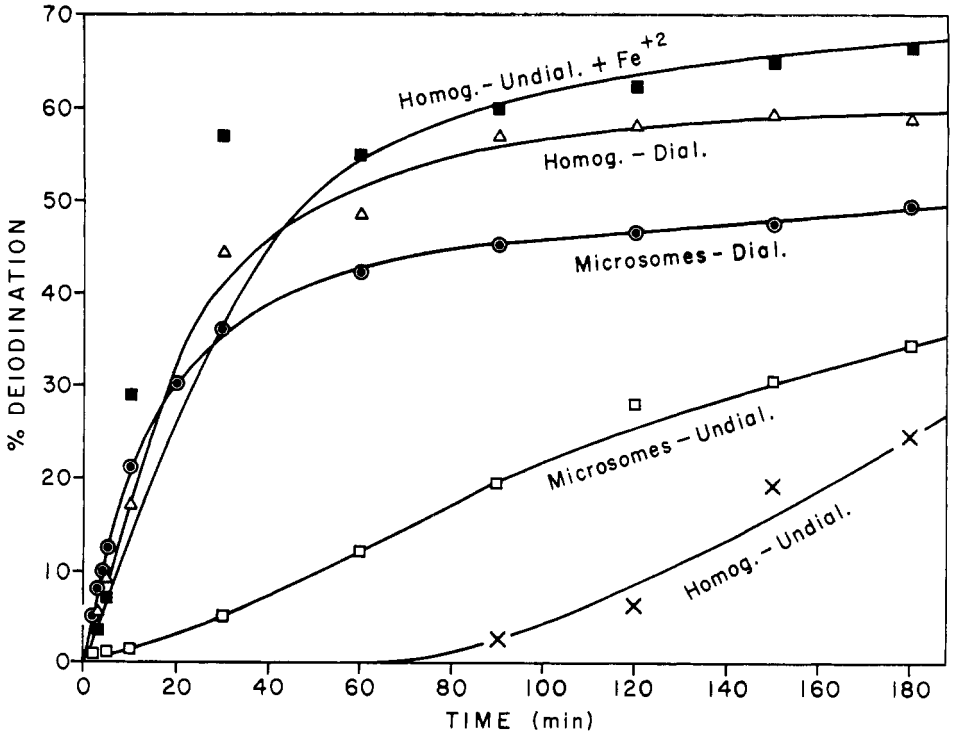

FIGURE 3: Effect of dialysis on T_4 deiodination by liver homogenate and liver microsomes. Labeled T_4 (160 mμmoles) was incubated with the equivalent of 400 mg of fresh liver for the time intervals shown.

out under an atmosphere of nitrogen, deiodination was completely inhibited in both the dialyzed and undialyzed systems. However, incubation under nitrogen did not destroy deiodinase activity and the original activity could be demonstrated after reincubation in an atmosphere of either oxygen or air. Since no significant differences were found between the rates of deiodination in oxygen or air, all subsequent incubations were performed in air.

Heat Stability of the Enzyme. The heat stability of the deiodinase enzyme was determined by heating various preparations in a boiling water bath for varying periods of time. Deiodinase activity in undialyzed homogenates was not readily inactivated by heat, and nearly 50% of the activity remained after boiling for 10 min (Figure 2A). Ferrous ions made the enzyme even more heat stable, but the enzyme in dialyzed liver homogenate and in undialyzed liver microsomes was quite heat liable (Figure 2B).

Time-Course Studies. A time lag of variable duration was generally encountered before T_4 and T_3 deiodination by undialyzed tissue homogenates assumed a linear rate. The lag period suggested the presence of an inhibitor since it could be completely overcome by dialysis of the tissue homogenate prior to incubation, or by the addition of an optimum concentration of ferrous ions to the incubation mixture. Following either treatment, deiodination proceeded at a rapid rate and remained linear for approximately 20 min (Figure 3). The lag phase with crude liver microsome preparations was always shorter than that observed with undialyzed homogenates, perhaps because some of the natural inhibitor was removed or inactivated during isolation of the microsomes. The lag phase observed for both T_4 and T_3 deiodination could be effectively eliminated by dialysis of the microsome preparation. Although ferrous ions also eliminated the lag phase seen with undialyzed homogenates, their addition to microsome preparations and dialyzed homogenates produced very erratic results.

Enzyme Kinetic Studies. TEMPERATURE DEPENDENCE. The effects of temperature on T_4 and T_3 deiodination by dialyzed liver and kidney homogenates were determined by incubating 160 mμmoles of substrate with 400 mg of liver homogenate in pH 5.8 phosphate buffer at different temperatures for 10 min. Under these conditions, the temperature optimum was rather sharp and reached a peak at 32–37°.

pH DEPENDENCE. The optimum pH for T_4 and T_3 deiodination was determined on a pooled sample of undialyzed liver homogenate, using four different buffers. The pH optimum was found to be the same for both substrates and was equal to approximately 5.8 in either Ringer-phosphate or phosphate buffer. Tris-maleate buffer gave a much broader curve, and citrate buffer inhibited deiodination at all pH values studied (Figure 4A). When the same study was repeated in buffer containing 1.7×10^{-3} M ferrous ions, the pH optimum for T_4 deiodination in phosphate buffer remained 5.8 and the inhibitory action of

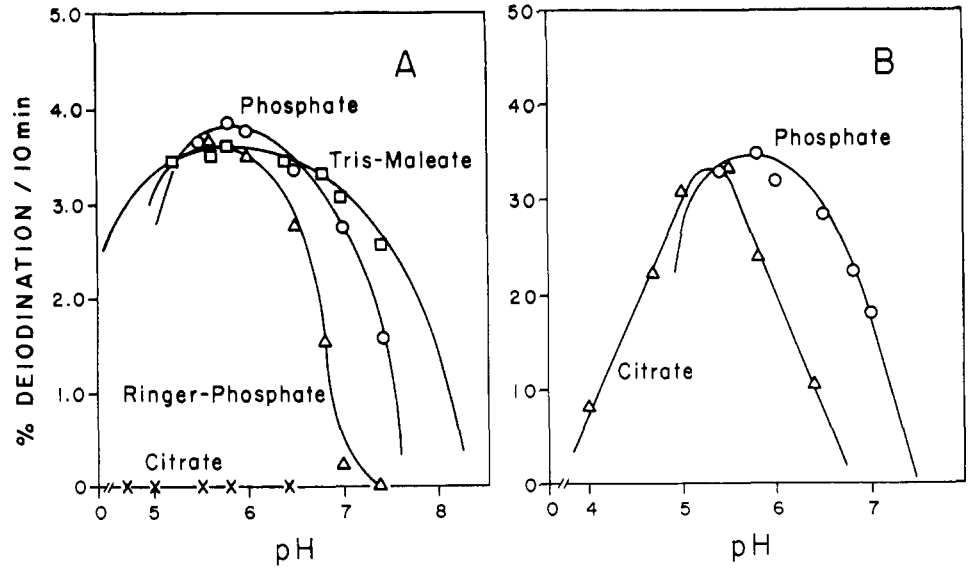

FIGURE 4: pH dependence of T_4 deiodination by undialyzed liver homogenate prepared in 0.1 M Ringer phosphate, phosphate, citrate, and Tris-maleate buffers (A) and 0.1 M phosphate and citrate buffers containing 1.7×10^{-3} M Fe^{2+} (B). Labeled T_4 (160 mμmoles) was incubated with 400 mg of liver at 37° and the hydrogen ion concentrations shown.

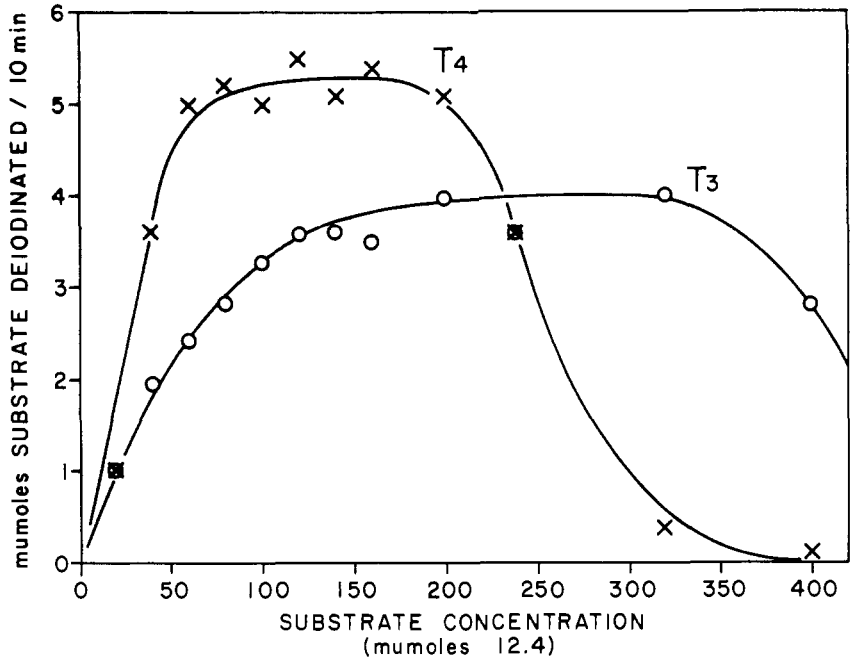

FIGURE 5: Substrate concentration dependence of T_4 and T_3 deiodination by 400 mg of undialyzed liver homogenate in pH 5.8 phosphate buffer.

citrate buffer was overcome. Therefore, a pH of 5.8 and phosphate buffer were used in all subsequent experiments, although neither dialyzed liver homogenate nor microsomes showed any pH dependence (pH 5.5–8.0) for either T_4 or T_3 deiodination.

SUBSTRATE DEPENDENCE. Aliquots of 400 mg of undialyzed liver homogenate were incubated with various substrate concentrations (Figure 5). Since a lag phase was generally encountered (as shown in Figure 3), the reaction velocity was calculated as the number of millimicromoles of substrate deiodinated in 10 min during the linear portion of the deiodination

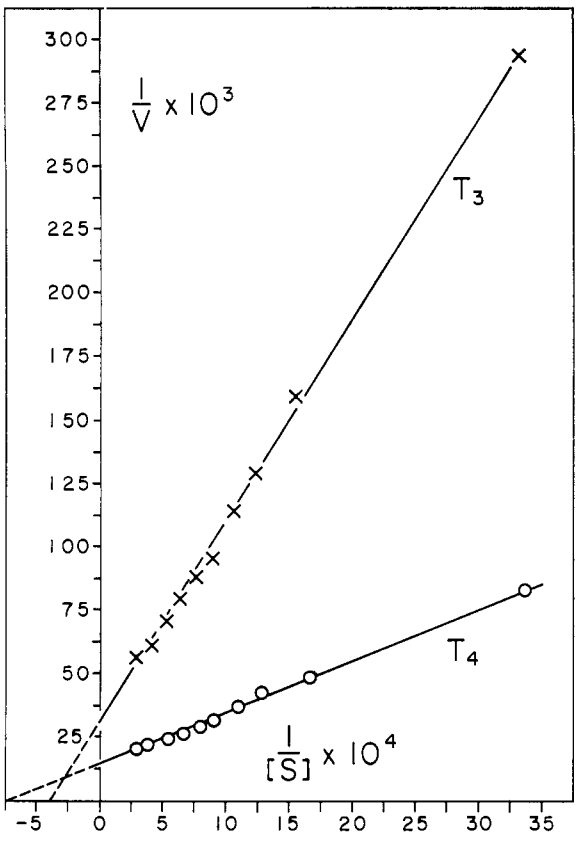

FIGURE 6: Substrate concentration dependence of T_4 and T_3 deiodination by 400 mg of dialyzed liver homogenate in pH 5.8 phosphate buffer. The reciprocals of the initial velocities (mμmoles of substrate deiodinated/10 min) have been plotted against the reciprocals of the substrate concentrations (molar concentration) in order to show the obeyance of Michaelis–Menton kinetics and to show the relationship between the K_s and V_{max} values for T_4 and T_3 as substrates.

time curve. The length of the lag period was not affected appreciably by increasing substrate concentration, and the reaction velocities shown in Figure 5 represent small but actual increases in the reaction velocity once the lag period was past. Substrate concentrations greater than 200 mμmoles of T_4 and 320 mμmoles of T_3 resulted in substrate inhibition.

When comparable amounts of dialyzed liver homogenate were used, substrate inhibition was not encountered and the initial reaction velocity was greatly increased. The inhibitor-free system obeys the Michaelis–Menten enzyme kinetic theory since a straight-line relationship was obtained when reciprocals of the initial velocity were plotted against reciprocals of the substrate concentration (Figure 6). Furthermore, the deiodinase enzyme had twice the affinity for T_4 ($K_s = 1.4 \times 10^{-5}$ M T_4) than for T_3 ($K_s = 2.7 \times 10^{-5}$ M), and the V_{max} for T_4 deiodination (66 mμmoles/10 min) was twice that for T_3 deiodination (31 mμmoles/10 min).

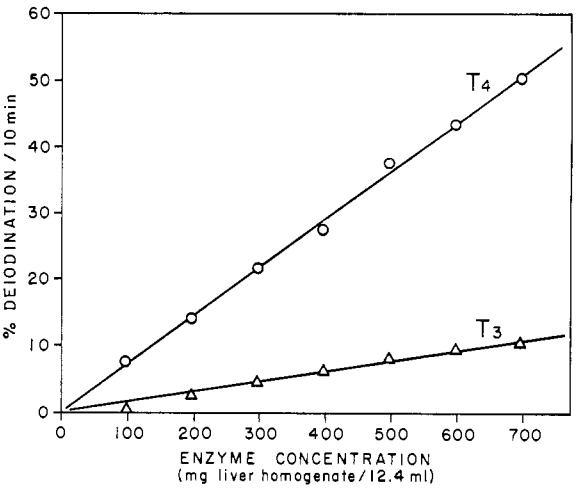

FIGURE 7: Enzyme concentration dependence of T_4 and T_3 deiodination by dialyzed liver homogenate. Labeled substrate (160 mμmoles) was incubated at 37° and pH 5.8 with the concentrations of liver homogenate shown.

ENZYME CONCENTRATION. In this series of experiments, a constant amount of substrate (160 mμmoles) was incubated with different amounts of dialyzed liver homogenate in 12.4 ml of pH 5.8 phosphate buffer. Good linear relationships were found between the initial velocity of the reaction and the enzyme concentration (Figure 7).

Optimum Conditions for Deiodination. Since a maximum of 60–70% deiodination was observed with T_4 and 30–35% with T_3, it was questioned whether these values were due to loss of enzyme activity, product inhibition, or approach toward equilibrium. To study these possibilities, 160 mμmoles of T_4 was incubated with 400 mg of dialyzed liver for 90 min, after which either 400 mg of fresh dialyzed liver homogenate or another 160 mμmoles of T_4 substrate was added to the incubation vessel. As shown in Figure 8 the addition of fresh enzyme produced no further increase in deiodination, whereas the addition of fresh substrate produced essentially the same amount of deiodination as before. Therefore, the enzyme must remain active throughout the usual incubation period, and the reaction is probably not inhibited appreciably by product formation. Rather, the maximum of 60–70% T_4 deiodination probably represents the approach toward equilibrium.

^{131}I-Labeled MIT and DIT as Substrates. Both ^{131}I-labeled MIT and DIT (160 mμmoles/reaction vessel) were also tested as potential substrates in the deiodinase system already described. The reaction was stopped by heating the reaction vessels in a boiling water bath for 2 min because the iodotyrosines are neither bound to plasma proteins nor precipitated by TCA. It was found that neither MIT nor DIT were suitable substrates for this system, and the unreacted labeled compounds could be recovered quantitatively from paper chromatograms.

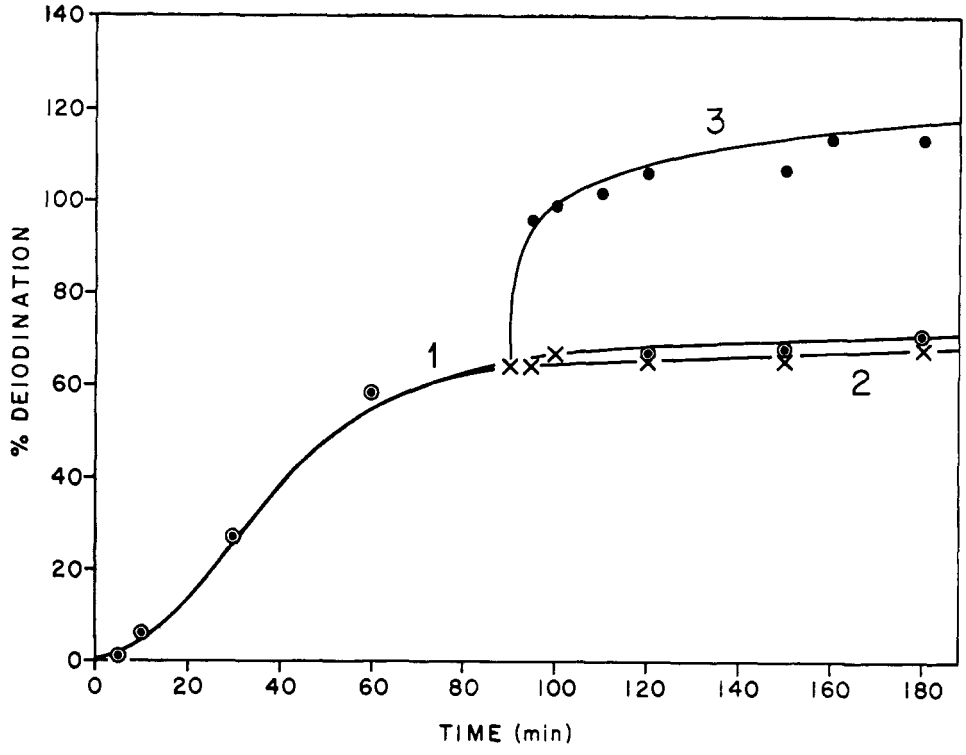

FIGURE 8: Effects of adding fresh enzyme (curve 2) and an additional 160 mμmoles of [^{131}I]T_4 (curve 3) to an incubation medium that had already been incubated for 90 min (curve 1). The original incubation mixture contained 160 mμmoles of T_4 and 400 mg of dialyzed liver homogenate, and incubation was carried out at 37° and pH 5.8.

Cell Fractionation Studies. Deiodinase activity was detected in all of the particulate fractions of the cell, but the highest enzyme concentration was always found in the microsomal fraction and no activity was ever detected in the cell sap (Table I). It was suspected that the nuclear and mitochondrial fractions were contaminated with microsomes and that deiodinase activity might be confined exclusively to the microsomes. Two types of experiments were performed to test this possibility. In the first, both the nuclear and mitochondrial fractions were washed repeatedly with 0.25 M sucrose, and the fractions and washes were reassayed for deiodinase activity. Since a considerable amount of activity was removed from the mitochondrial and nuclear fractions with repeated washing, the activity seen in these fractions was probably due to microsomal contamination. Repeated washing of the microsomal fraction produced no loss in activity, and some increase in specific activity. However, the deiodinase activity found in several subfractions generally exceeded the activity in the original homogenate. The probable explanation is that the tissue deiodinase inhibitor became diluted with repeated washings, thereby causing an increase in deiodinase activity.

In the second type of experiment, microsomal contamination was determined by measuring the amount of glucose 6-phosphatase activity in the mitochondrial and microsomal fractions (Harper and Bergmeyer, 1963). This enzyme is found only in microsomes (De Duve *et al.*, 1949), and its presence in the mitochondrial fraction indicates microsomal contamination. It was found that the unwashed mitochondrial fraction contained sufficient glucose 6-phosphatase activity to yield 1.36 μmoles of inorganic phosphate/15 min. The microsomal preparation gave a value of 2.64 μmoles of phosphate/15 min, and the ratio of mitochondrial:microsomal phosphate produced was 0.52

TABLE I: Distribution of T_4 and T_3 Deiodinase Activity in Subcellular Fractions of Dialyzed Rat Liver Homogenate.

Cell Fraction	T_3 Enzyme Act.[a]	Sp Act.	T_4 Enzyme Act.[a]	Sp Act.
Homogenate	12.7	0.57	24.2	1.00
Nuclei	7.0	1.05	16.2	2.42
Mitochondria	4.6	0.74	10.3	1.65
Microsomes	10.9	2.05	18.0	3.38
Cell sap	0.0	0.0	0.0	0.0

[a] Enzyme activity is expressed as % of the substrate (160 mμmoles) deiodinated/10 min per 400 mg of fresh tissue, and specific activity is defined as % of the substrate deiodinated/10 min per mg of protein.

(1.36:2.64). Since a ratio of mitochondrial:microsomal T_4 deiodination (Table I) was found to be 0.57 (10.3:18.0), it is probable that the unwashed mitochondrial preparation was highly contaminated with microsomes and that little if any deiodinase activity actually resided in the mitochondria.

Solubilization of Microsomal Deiodinase. Since the microsomal fraction contained the largest concentration of deiodinase activity per milligram of protein, attempts were made to solubilize the microsomal enzyme by sonication, deoxycholate, and butanol treatment. When microsomal preparations were sonicated in a refrigerated Raytheon 9-kcycle sonicator for 50 min, 23.2% of the T_4 deiodinase activity appeared in the soluble fraction, and the enzyme remained completely stable. In samples sonicated for 150 min, 33% of the enzyme became solubilized.

Microsomal preparations incubated at 2° and pH 5.8 for 4 hr with 1% deoxycholate resulted in 18% solubilization of the enzyme, and the enzyme remained completely stable. The butanol solubilization technique (Morton, 1955) was unsuccessful since a large per cent of the enzyme became inactivated.

Tissue Distribution of the Enzyme. Both dialyzed and undialyzed homogenates of the major tissues of the rat were assayed for T_4 and T_3 deiodinase activity (Table II). Liver, kidney, and heart contained the highest concentration of T_4 deiodinase, whereas testis, liver, and heart were highest in T_3 deiodinase activity. Undialyzed thyroid and adrenal homogenates were completely inactive, but dialyzed homogenates were not tested because of insufficient tissue. Appreciable amounts of T_4 deiodinase inhibitor were present in all of the major tissues, as evidenced by a marked increase in deiodinase activity following dialysis. On the other hand, only liver, kidney, heart, spleen, and testis contained significant amounts of T_3 inhibitor.

TABLE II: Relative Activities of Dialyzed and Undialyzed Rat Tissue Homogenates for T_4 and T_3 Deiodination.

	% Substrate Deiodinated/10 min per 400 mg of Tissue[a]			
	T_3		T_4	
Tissue	Undialyzed	Dialyzed	Undialyzed	Dialyzed
Liver	3.3	9.3	3.0	19.1
Kidney	0.5	3.4	0.8	10.1
Intestine	1.7	1.0	0.7	3.9
Heart	1.4	5.0	1.4	21.2
Spleen	1.8	3.7	0.3	2.6
Brain	0.7	0.4	0.9	4.3
Skeletal muscle	0.5	0.6	0.8	5.0
Testis	2.5	20.2	1.0	5.3
Thyroid	0	—	0	—
Adrenal	0	—	0	—

[a] Labeled T_4 or T_3 (160 mμmoles) was incubated with 400 mg of homogenized fresh tissue for sufficient times to allow for the calculation of initial reaction rates. Incubations were carried out at 37° and pH 5.8.

Activators and Inhibitors. The effects of potential enzyme activators and inhibitors were studied on both the inhibitor-free (dialyzed) and inhibitor-containing (undialyzed) T_4 deiodinase systems. Controls were run in each of the separate experiments, and the results are expressed as per cent of control dialyzed deiodinase activity (Tables III and IV). Thus, the effects of activators and inhibitors directly on the enzyme (dialyzed preparation) can be separated from those effects observed on the inhibited system.

EFFECTS ON THE DIALYZED ENZYME. The major inhibitors of T_4 deiodinase activity were (a) cyanide, (b) *p*-mercuribenzoate, cystine, and the metals (Hg^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}), (c) hydrogen sulfide, (d) serotonin and 5-hydroxytryptophan, and (e) NADH, NADPH, and ascorbate.

Results with the potential sulfhydryl blocking agents suggest that sulfhydryls might be involved at the active site of the enzyme. However, the inhibitory actions of the hydroxylated compounds (serotonin and 5-hydroxytryptophan) and certain reducing substances (NADH, NADPH, and ascorbate) appear to be equally as important. Hydrogen sulfide gas, bubbled into the reaction medium for only 30 sec, was completely inhibitory. This result might suggest (a) the need for a metal ion, although EDTA and citrate (both metal chelating agents) produced no inhibition, or (b) that the reaction does not take place in the presence of a strong reducing agent.

Of the substances tested, EDTA, thioglycolate, oxidized glutathione, pyridoxyl phosphate, and the metal ions (Fe^{3+}, V^{2+}, Mg^{2+}, Cd^{2+}, and Cr^{2+}) stimulated the deiodination reaction. The amount of stimlation was variable, but thioglycolate, oxidized glutathione, pyridoxal phosphate, and Fe^{3+} more than doubled the reaction rate.

EFFECTS ON THE NATURAL INHIBITOR SYSTEM. Many of the substances tested either produced further inhibition of the undialyzed deiodination system or were ineffective in counteracting the tissue inhibitor. Substances which inhibited the dialyzed enzyme also produced further inhibition in the undialyzed system. Examples of these include cyanide, the sulfhydryl blocking agents (*p*-mercuribenzoate and mercuric chloride), serotonin and 5-hydroxytryptophan, and the reducing substances (NADH, NADPH, and ascorbate). In addition, the metal chelating agents (EDTA, citrate, and ascorbate) and some of the metal ions (Cd^{2+}, Cr^{2+}, and Pb^{2+}) were quite inhibitory in the undialyzed system. The only stimulatory substances were thioglycolate and certain metal ions (V^{2+}, Fe^{2+}, and Fe^{3+}). Thus it appears that the actions of inhibitors and activators on the deiodinase system can be separated from those on the natural inhibitor system.

Properties of the Tissue Deiodinase Inhibitor. The time lag and suboptimal velocity of the deiodination

TABLE III: Effects of Various Substances on T_4 Deiodination by Undialyzed and Dialyzed Liver Homogenates.[a]

Compd	Concn (M)	Undialyzed	Dialyzed	Compd	Concn (M)	Undialyzed	Dialyzed
Control		12	100	H_2S		6	0
KCN	8.3×10^{-4}	5	13	Pyridoxal phosphate	6.7×10^{-4}	0	114
					6.7×10^{-3}	0	233
Sodium azide	2.1×10^{-4}	19	97	Serotonin	6.6×10^{-5}	0	48
	1.7×10^{-3}	19	93		1.3×10^{-3}	0	4
H_2O_2	2.3×10^{-4}	17	110	Tryptophan	4.2×10^{-4}	17	100
	1.5×10^{-3}	22	107		8.3×10^{-3}	9	70
Catalase	6×10^4 units	12	90	5-Hydroxy-tryptophan	8.3×10^{-5}	2	66
EDTA	2.1×10^{-4}	0	93		1.7×10^{-3}	0	0
	2.5×10^{-3}	0	117	Tryptamine	8.3×10^{-5}	14	79
	2.5×10^{-2}	0	149		1.7×10^{-3}	0	64
Citrate	8.3×10^{-5}	12	100	NAD^+	1.7×10^{-3}	13	81
	1.7×10^{-3}	0	108				
Isocitrate	8.3×10^{-5}	12	104	NADH	1.1×10^{-3}	2	17
	1.7×10^{-3}	12	125	NADP	1.0×10^{-3}	12	92
β-Glucuronidase (2.5 units/mg)	2.0 mg	12	100	NADPH	8.8×10^{-4}	2	17
	5.0 mg	12	97				
	10.0 mg	—	86				
PMB	2.1×10^{-4}	25	117	FAD	8.7×10^{-4}	12	107
	1.7×10^{-3}	8	66	FMN	1.7×10^{-3}	14	111
	6.6×10^{-3}	4	27				
				ATP	1.7×10^{-3}	17	112
$HgCl_2$	1.7×10^{-3}	4	13				
				ADP	1.7×10^{-3}	13	93
Cysteine	8.3×10^{-5}	10	83				
	1.7×10^{-3}	7	67	CTP	5.8×10^{-3}	12	83
	8.3×10^{-3}	7	58				
				GTP	6.3×10^{-4}	15	84
Thioglycolate	3.3×10^{-4}	44	326				
				ITP	1.4×10^{-3}	14	90
GSH	6.6×10^{-5}	12	82	Ascorbic acid	8.3×10^{-5}	12	100
	1.3×10^{-3}	6	60		4×10^{-4}	0	50
					8.3×10^{-4}	0	22
GSSG	6.6×10^{-5}	14	98		4×10^{-3}	0	5
	1.3×10^{-3}	12	222				

[a] All values are relative to control dialyzed activity, which was assigned an arbitrary value of 100. Initial velocities from which the relative values were computed were obtained by incubating 160 mμmoles of labeled T_4 with 400 mg of dialyzed liver homogenate in the presence of the substances listed. Incubations were carried out at 37° and pH 5.8. Abbreviations used: GSH and GSSG, glutathione and oxidized GSH; NAD^+, oxidized nicotinamide–adenine dinucleotide; PMB, *p*-mercuribenzoate; NADP, nicotinamide–adenine dinucleotide phosphate; ATP, CTP, GTP, and ITP, adenosine, cytidine, guanosine, and inosine triphosphates.

reaction can be overcome by dialysis of the enzyme preparation prior to incubation. However, it is possible that the deiodinase inhibitor becomes inactivated and not actually removed during overnight dialysis. In order to determine whether aging would activate the undialyzed preparation, a pooled liver homogenate was divided into two parts. One portion was dialyzed against distilled water at 5° and the other was held at 5° for the same period of time (Table V). The lag period was shortened by aging, but T_4 deiodinase activity remained unaffected after 1 day. Although a threefold increase in activity was observed after 3 days of aging, nearly a sevenfold increase in deiodinase activity was seen after 1 day of dialysis. T_3 deiodinase activity was not increased with aging, although the lag period was reduced. Therefore, the inhibitor must dissociate from the enzyme and be removed by dialysis since the enzyme was activated much more rapidly

TABLE IV: Effects of 1.7×10^{-3} M of Various Cations on T_4 Deiodination by Undialyzed and Dialyzed Liver Homogenate.

Metal	% Dialyzed Control Act.[a]	
	Undialyzed	Dialyzed
Control	10	100
Fe^{2+}	60	14
Fe^{3+}	24	211
V^{2+}	109	178
Co^{2+}	2	52
Cu^{2+}	0	4
Zn^{2+}	2	54
Hg^{2+}	3	13
Mn^{2+}	3	90
Ca^{2+}	8	90
Mg^{2+}	8	124
Cd^{2+}	0	149
Cr^{2+}	0	149
Pb^{2+}	0	116

[a] The experimental conditions were the same as those cited in Table II.

and effectively by dialysis than by aging. It also appears that the inhibitor protects the enzyme from inactivation since the addition of ferrous ions to a 3-day-old homogenate produced a much faster rate of deiodination than that of a 3-day-dialyzed preparation.

It was observed earlier that the time lag in deiodination can be eliminated by preincubation of the reaction mixture prior to the addition of substrate. A further study was made by preincubating liver homogenate at 37° for varying periods of time prior to the addition of T_4 substrate. The lag period was eliminated after 10 min, and 60 min of preincubation gave rise to a maximum rate of deiodination. However, the deiodinase activity of preincubated homogenate was considerably lower than that of dialyzed preparations.

Since dialysis is an effective method for eliminating the inhibitor, it was thought that dialysates of liver homogenates might contain the inhibitor. To test this possibility, 120 ml of 40% liver homogenate prepared in glass-distilled water was dialyzed against 5 l. of glass-distilled water for 24 hr at 5° and the dialysate was concentrated to a final volume of 50 ml by lyophilization. Increasing amounts of the concentrated dialysate produced correspondingly greater inhibition of dialyzed enzyme when either T_4 and T_3 was used as substrate (Figure 9). The concentrated dialysate reduced both the rate and maximum amount of substrate deiodinated, but it did not produce a lag period like that seen with the natural tissue inhibitor.

Preliminary studies were also made of the properties of the dialysate inhibitor. It was not inactivated by boiling for 10 min nor by bubbling oxygen through it for 10 min (Figure 10). Preincubation of the dialyzed enzyme with dialysate factor produced only a slight increase in inhibitory action, and the addition of 1.7×10^{-3} M ferrous ions did not overcome the inhibition. Since a lag phase was not produced and since ferrous ions were ineffective, the dialysate inhibitor may be different from the natural tissue inhibitor. It might be due to some metal ion or other dialyzable substance that was concentrated during the dialysis procedure.

Since the natural inhibitor appears to be water soluble and dialyzable, it might be found in the cell sap (the 105,000g supernatant fraction). When 4 ml of cell sap was added to dialyzed liver homogenate (final volume 12.4 ml), neither a time lag nor a decrease in the velocity of deiodination was noted. However, if the cell sap was preincubated for 15 min with the

TABLE V: Relative T_3 and T_4 Deiodinase Activities in Dialyzed or Aged Liver Homogenate, in the Presence and Absence of an Optimal Concentration of Ferrous Ions.[a]

Dialyzing Time (days)	Fe^{2+} Concn (M)	% Substrate Deiodinated/10 min		Aging Time (days)	T_3		T_4	
		T_3	T_4		% Deiodinized/10 min	Lag Phase (min)	% Deiodinized/10 min	Lag Phase (min)
0	0	4.2	5.0	0	4.2	30	5.4	30
	1.7×10^{-3}	7.2	37.6		7.2	10	37.6	10
1	0	11.9	33.4	1	3.5	7	4.3	7
	1.7×10^{-3}	4.2	11.8		20.5	10	44.2	8
3	0	4.3	13.9		3.9	0	15.6	0
				3				
	1.7×10^{-3}	2.8	17.6		26.3	10	44.2	7

[a] Labeled T_3 or T_4 (160 mμmoles) was incubated at 37° and pH 5.8 with the equivalent of 400 mg of homogenized liver that had been either dialyzed or aged at 5° for the times shown. Sufficient experimental points were taken to establish the length of the lag phase and to determine the initial reaction rate.

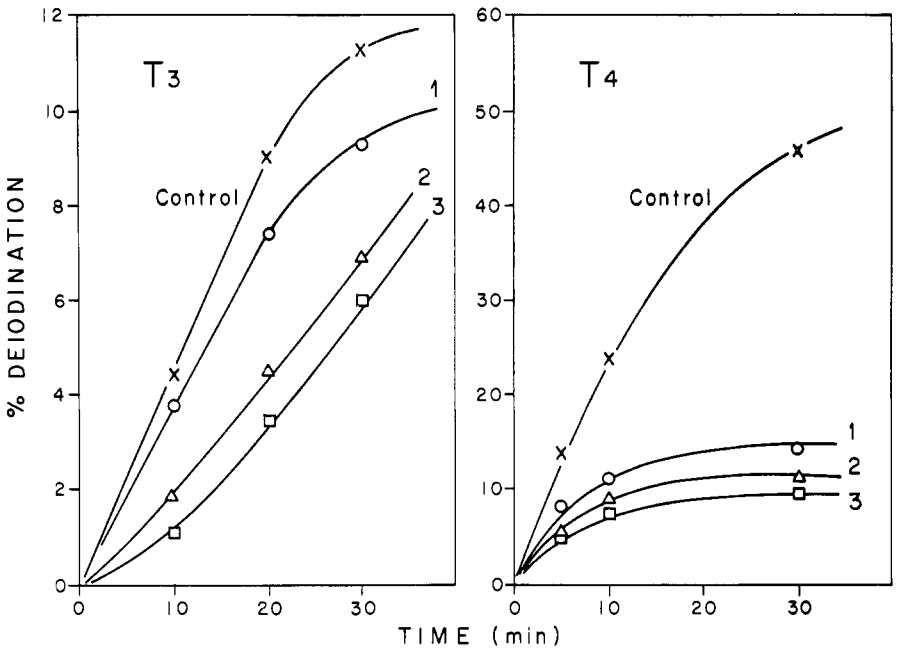

FIGURE 9: Inhibitory effect of increasing amounts of liver dialysate on dialyzed liver homogenate activity. The volumes of concentrated dialysate summarized below were added to incubation media containing 160 mμmoles of labeled T_4 or T_3 and the equivalent of 400 mg of dialyzed liver homogenate. Incubations were carried out at 37° and pH 5.8 and a final volume of 12.4 ml. (T_3 deiodination) Curves 1–3 represent T_3 deiodination in the presence of 3–5 ml of the concentrated dialysate. (T_4 deiodination) Curves 1–3 represent T_4 deiodination in the presence of 4–6 ml of concentrated dialysate.

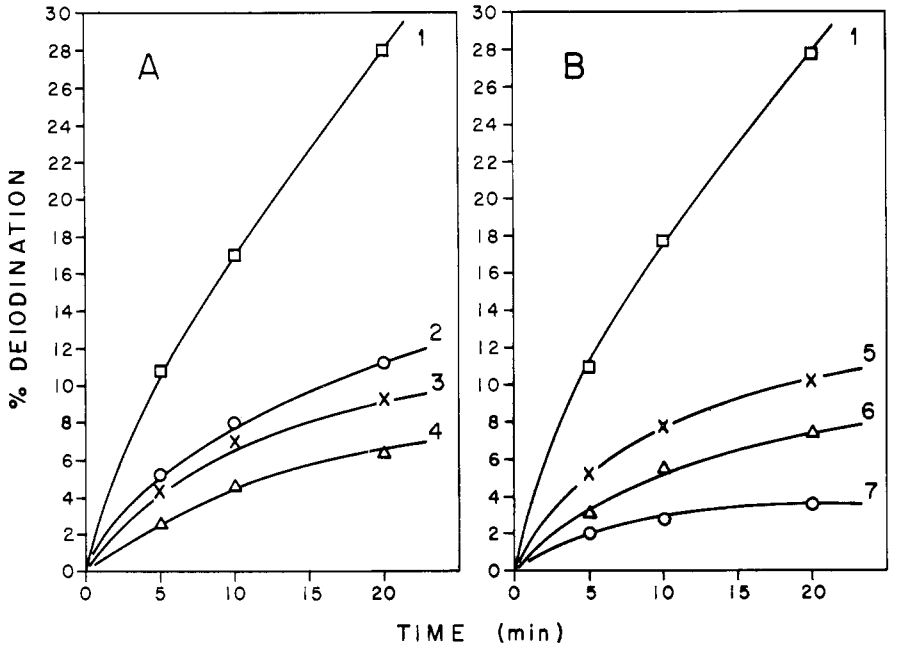

FIGURE 10: Stability of the inhibitor found in concentrated dialysate. After the treatment summarized below, 4 ml of the treated dialysate was added to the incubation medium containing 160 mμmoles of labeled T_4 and 400 mg of dialyzed liver homogenate. Incubations were carried out at 37° and pH 5.8 in a final volume of 12.4 ml. After various treatments, 4 ml of treated dialysate was added to the incubation medium containing dialyzed liver homogenate: (1) control, without dialysate; (2) untreated dialysate; (3) oxygen bubbled through the dialysate for 10 min; (4) dialysate boiled for 10 min; (5) untreated dialysate; (6) dialysate and the dialyzed liver homogenate preincubated for 5 min; (7) dialysate plus 1.7×10^{-3} M ferrous ions.

TABLE VI: Effects of Thyroidectomy and Hyperthyroidism on the Deiodinase Activity of Several Rat Tissues.[a]

Tissue	Thyroidectomized		Euthyroid		Hyperthyroid	
	Undialyzed	Dialyzed	Undialyzed	Dialyzed	Undialyzed	Dialyzed
Liver	1.2 ± 0.2 (12)	21.7 ± 2.1 (14)	3.2 ± 0.1 (11)	27.0 ± 3.6 (13)	3.4 ± 0.4 (11)	22.4 ± 2.4 (13)
Heart	0.4 ± 0.1 (14)	16.8 ± 1.9 (18)	0.7 ± 0.1 (10)	16.6 ± 2.8 (12)	2.6 ± 0.1 (10)	18.0 ± 1.5 (12)
Kidney	0.6 ± 0.2 (6)	9.8 ± 2.1 (6)	0.5 ± 0.2 (6)	6.5 ± 1.4 (6)	1.3 ± 0.2 (6)	8.0 ± 1.4 (6)
Skeletal muscle	0.6 ± 0.1 (6)	1.2 ± 0.2 (6)	0.9 ± 0.2 (6)	1.3 ± 0.2 (6)	1.3 ± 0.3 (6)	2.6 ± 0.2 (6)

[a] The number of animals used is given in parentheses and the deiodinase activity is given as the mean plus or minus the standard error. The units of deiodinase activity are % substrate deiodinated/10 min.

dialyzed homogenate before the addition of substrate, a lag period of 15 min and an 80% loss in deiodinase activity were observed. Therefore, the natural inhibitor appears to be present in the cell sap in easily detectable amounts.

Deiodinase and Inhibitor Concentrations in Hypo-, Hyper-, and Euthyroid Rats. A study was undertaken to determine whether tissue deiodinase activity is under the control of thyroid hormone. One group of intact euthyroid rats served as controls, a second group of intact rats was made hyperthyroid by daily subcutaneous injections of 40 μg of L-T_4/100 g of body weight day for 3 weeks, and a third group consisted of surgically thyroidectomized animals. At the end of 3 weeks, the animals were sacrificed by decapitation and the homogenates of several major tissues (before and after dialysis) were assayed for deiodinase activity. It was found that the level of thyroid hormone activity had no effect upon the tissue levels of deiodinase (Table VI), and that thyroid hormone did not induce the synthesis of additional deiodinase enzyme. On the other hand, the tissues of thyroidectomized animals contained more inhibitor than those of hyperthyroid animals, as evidenced by the lower deiodinase levels seen in undialyzed tissue homogenates of thyroidectomized animals.

Thiouracil Effects. Whether or not Tu inhibits T_4 deiodination *in vitro* and *in vivo* is still controversial. In the present study, a relatively high *in vitro* concentration of Tu (1.25×10^{-2} M) was required to inhibit the deiodination of T_4 by 50%, and no time lag was observed. When Tu was given in the drinking water (50 mg/100 ml) or fed at a level of 0.2% of the diet for 14 days, it had little or no effect on liver T_4 deiodinase activity. From these observations, it is concluded that physiological amounts of Tu do not significantly interfere with T_4 deiodination.

Discussion

There seems little doubt that B-ring deiodination of T_4 and T_3 by rat liver is enzymatic in nature and that deiodinase activity in crude tissue homogenates is largely blocked by a naturally occurring inhibitor. The presence of such an inhibitor is probably responsible for much of the confusion that has surrounded the deiodination reaction. For example, a time lag of varying duration was always observed before the reaction velocity could be measured, and even then the reaction velocity was always suboptimal (Figure 3). The inhibitor also appeared to be responsible for the relative heat stability, pH dependence, and substrate inhibition of the system because all three effects disappeared after the inhibitor was removed by dialysis. The heat stability of deiodinase preparations has been reported by others (Stanbury *et al.*, 1960; Yamamoto, 1964; Lissitzky *et al.*, 1956; Galton and Ingbar, 1966), and it has been used to challenge the enzymatic nature of the system. However, in view of the marked heat instability of the dialyzed enzyme, it is probable that the inhibitor was responsible for the heat stability observed in crude enzyme preparations.

The same concentration of deiodinase enzyme was found in any given tissue (except skeletal muscle) in thyroidectomized, euthyroid, and hyperthyroid rats. However, the inhibitor concentration was highest in thyroidectomized animals and lowest in hyperthyroid animals. These results are in apposition to those of Larson *et al.* (1955) who stated that increased deiodinase activity in hyperthyroid rats was due to an adaptive enzyme response to T_4. However, these workers were probably unaware of the deiodinase inhibitor and did not recognize that changes in deiodinase activity are the result of changes in inhibitor concentration.

Although the observations were complicated somewhat by changing concentrations of the natural inhibitor, it would appear that deiodinase activity is highest in liver, kidney, and heart tissue, and that it is present in highest concentrations in the microsomal compartment of the cell. The natural inhibitor, on the other hand, appears in highest concentration in the soluble fraction of the cell, and is dialyzable. A concentrated

dialysate from fresh liver homogenate inhibited deiodination, but it is believed that this inhibition might have been due to some other substance that was concentrated during the processing of the dialysate. One reason for this belief is that the concentrated dialysate inhibitor failed to show a lag phase comparable to that produced by the addition of cell sap or to that seen in the dialyzed system.

Yamamoto (1964) suggested that ascorbate might be a naturally occurring inhibitor of deiodinase activity. He found that undialyzed enzyme was relatively stable to heat and that adding ascorbate to the crude system further protected it from heat inactivation. He pointed out that rat liver contains 10^{-3} M ascorbate and speculated that this concentration of ascorbate might well acccount for the inhibition observed. The observations made in the present study tend to support this concept. For example, an ascorbate concentration of 10^{-3} M produced marked inhibition in the *in vitro* deiodinase system, although ascorbate did not produce a lag period like that seen with the natural inhibitor. Furthermore, Fe^{2+} and V^{2+} ions activated the inhibitor-containing system, possibly by chelating with ascorbate or by acting in conjunction with oxygen to destroy the ascorbate. Since the deiodination reaction is an oxidative one (an absolute oxygen requirement was demonstrated), reducing substances such as ascorbate might interfere with the oxidative mechanism. However, other reducing substances such as NADH, NADPH, cysteine, 5-hydroxytryptophan, reduced glutathione, and serotonin also inhibited the reaction. T_4 itself is a good reducing substance with an oxidation–reduction potential of -0.8 (pH 0), and its oxidation to the corresponding quinone may be a prerequisite for deiodination. Therefore, certain reducing substances might compete with T_4 for the electron acceptor group at the active site on the enzyme. Such a group would have to be continually reoxidized in order to accept electrons from T_4 and a potent reducing substance such as ascorbate might keep the electron acceptor in the reduced form. It has also been reported that ascorbic acid can reduce quinones to hydroquinones nonenzymatically (Sanadi, 1964). Since T_4 is a hydroquinone, ascorbate might also keep the T_4 molecule in a reduced form, thereby preventing its oxidation to the quinone form with subsequent deiodination. If deiodination proceeds by oxidation of the phenol group, cleavage at the ether bridge might be anticipated. Plaskett (1961), Wynn and Gibbs (1962, 1964), and Benevent *et al.* (1963) have presented evidence for such a mechanism and have demonstrated the existence of bound diiodotyrosine following deiodination.

The only substances that stimulated dialyzed liver deiodinase activity were thioglycolate, oxidized glutathione, pyridoxal phosphate, and several metal ions. It is difficult to know whether these substances act nonspecifically or whether they might be considered as potential cofactors in the deiodination reaction. Although FMN and FAD were not required in the present system, Galton and Ingbar (1962) reported that both substances catalyzed the nonenzymatic photochemical deiodination of T_4 and T_3 when incubation was carried out in bright light. These authors also reported that the *in vitro* addition of azide to mouse liver homogenates simultaneously decreased catalase activity and increased deiodinase activity (Galton and Ingbar, 1964). However, catalase, hydrogen peroxide, and azide had no effect upon deiodinase activity in the present study. Some stimulation was seen in the undialyzed system, but the increase was small compared to the eightfold increase produced by dialysis.

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