Iodothyronine 5'-Deiodinase in Rat Kidney Microsomes

Kinetic Behavior at Low Substrate Concentrations

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Abstract. The thiol-activated enzymatic outer-ring monodeiodination of iodothyronines by rat kidney microsomes at low (nanomolar) substrate concentrations shows an apparently sequential reaction mechanism and is further characterized by insensitivity to inhibition by dicoumarol, a moderate sensitivity to inhibition by propylthiouracil (K_i = 100 μM) and iopanoic acid (K_i = 0.9 mM), responsiveness to 5 mM glutathione (GSH), and a thermal activation profile that is concave downward with a T_d of ~20°C. In contrast, the activity at high (micromolar) substrate concentrations shows a ping-pong reaction mechanism, is inhibited by micromolar concentrations of propylthiouracil, iopanoic acid and dicoumarol, is unresponsive to 5 mM GSH, and shows a concave upward thermal activation profile. Analysis of the microsomal deiodinase reaction over a wide range of 3,3',5'-triiodothyronine (rT_3) concentrations (0.1 nM to 10 μM) suggested the presence of two enzymatic activities, with apparent Michaelis constants (K_m) of 0.5 μM and 2.5 nM. Lineweaver-Burk plots of reaction velocities at nanomolar substrate concentrations in presence of 100 μM propylthiouracil also revealed an operationally distinct enzymatic activity with K_m's of 2.5 and 0.63 nM and maximum velocities (V_{max}'s) of 16 and 0.58 pmol/mg protein per h for rT_3 and thyroxine (T_4), respectively. These findings are consistent with the presence of a low K_m iodothyronine 5'-deiodinase in rat kidney microsomes distinct from the well characterized high K_m enzyme and suggest that at circulating levels of free T_4 the postulated low K_m enzyme could be physiologically important.

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Introduction

Whereas the thyroid gland is the sole source of circulating thyroxine (T_4),[1] 3,5,3'-triiodothyronine (T_3), the biologically active form of the thyroid hormone, is largely generated by 5'-monodeiodination of T_4 extrathyroidally. The enzyme system responsible for this conversion has been identified in the microsomal fractions of the rat liver and kidney and partially purified (1, 2). The microsomal enzyme has an apparent Michaelis constant (K_m) of ~5 μM for T_4, is activated by thiols, especially dithiols, in a two-step transfer (ping-pong) mechanism, is inhibited by propylthiouracil and can use reverse T_3 (3,3',5'-triiodothyronine, rT_3) as an alternate substrate (K_m ~ 0.5 μM) (3).

A low K_m (10^{-9} M for T_4) iodothyronine 5'-deiodinase has recently been described in rat brain (4), pituitary (5), and brown adipose tissue (6). The enzyme (designated type II) also uses rT_3 as an alternate substrate with high affinity (K_m ~ 3 × 10^{-9} M), but is different from the high K_m (type I) hepatic and renal enzyme in being insensitive to propylthiouracil (PTU) and in its thiol activation mechanism, which is believed to be sequential rather than ping-pong (5). This type II enzymatic pathway may be particularly important in regulation of intracellular, as opposed to circulating triiodothyronine levels, but may also play a role in the generation of that fraction of extrathyroidally produced T_3 that is not blocked by PTU in athyreotic T_4-replaced rats (7). Based on inhibition analysis with PTU, Silva and co-workers (7) also concluded that the type II pathway, while of major importance in brain and pituitary, was of little, if any, quantitative significance in the rat liver.

In the present study, we have examined the kinetic behavior of rat kidney microsomal iodothyronine 5'-deiodinase at low (nanomolar) concentrations of the substrates, T_4 and rT_3. The experiments revealed some properties of the enzyme at low substrate concentrations that differed significantly from those at high substrate concentrations and, indeed, resembled in

1. *Abbreviations used in this paper:* DHL, reduced lipoamide; DTT, dithiothreitol; GSH, glutathione; PTU, propylthiouracil; rT_3, 3,3',5'-triiodothyronine; T_3, 3,5,3'-triiodothyronine; T_4, thyroxine.

some, but not all, respects the characteristics described for the type II enzyme in the cerebral cortex, pituitary, and brown adipose tissue. We report here some observations on kidney microsomal 5'-deiodination of T_4 and rT_3 at low substrate concentrations and raise the possibility, on the basis of kinetic and other data, that rat kidney microsomes may contain both a low and a high K_m iodothyronine 5'-deiodinase.

Methods

Reagents. L-[3'- or 5'-^{125}I] rT_3 and T_4 (750–1,250 μCi/μg) were obtained from New England Nuclear (Boston, MA) and were further purified just before use by high voltage paper electrophoresis (3). Dihydrolipoamide (DHL) was prepared by borohydride reduction of the oxidized form (obtained from Sigma Chemical Co., St. Louis, MO) by the method of Reed et al. (8) as modified by Silver (9). Iopanoic acid (Telepaque) was a product of Winthrop Laboratories Co., New York; a stock solution was made by alkaline extraction of the tablets and neutralized before use.

Assay of microsomal iodothyronine 5'-deiodinase. Rat renal microsomes were prepared by methods as described earlier (10). The incubation medium contained, in a total volume of 200 μl: microsomal protein, 50 μg; K-phosphate buffer (pH 7.0), 20 μmol; EDTA, 200 nmol; and varying amounts of dithiothreitol (DTT), rT_3, or T_4 (containing ~50,000 cpm of ^{125}I-labeled rT_3 or T_4), as indicated in legends. The incubation times were 3 min for rT_3 (except in the experiment of Fig. 1) and 60 min for T_4. The incubations were terminated by the addition of 0.1 ml of 2% bovine serum albumin (BSA), followed immediately by 0.5 ml of 10% ice-cold trichloroacetic acid. $^{125}I^-$ liberated was measured after separation by ion-exchange chromatography on Bio-Rad Ag50WX8(H+) resin (BioRad Laboratories, Richmond, CA) as described previously (10). The reaction rates were expressed as picomoles of rT_3 degraded per milligram protein per hour. The products were frequently checked by descending paper chromatography (solvent system—hexane/*t*-amyl alcohol/ammonia (2 N) 1:10:11).

Results

Studies of microsomal 5'-deiodination over a wide range of substrate concentrations. Fig. 1 shows rT_3 deiodinase activities of kidney microsomes, at 5 mM DTT, from a group of normal rats and from rats given intraperitoneal PTU (2 mg/100 g body wt) 15 min before killing. A plot of enzyme reaction velocities vs.-log [*S*] for normal microsomes was sigmoid (Fig. 1 *A*), with an inflection at ~0.5 μM and a maximum velocity of ~58 nmol/mg protein per h, thus reflecting the well-documented characteristics of the kidney 5'-deiodinating enzyme. In a plot of the activities at substrate levels <10 nM and extending down to 0.1 nM on a 25-fold expanded scale, however, a deviation was noted between the observed reaction velocities and those predicted by the Michaelis equation, using values for K_m and V_{max} of 0.5 μM and 58 nmol rT_3 degraded/mg protein per h, respectively, as obtained from experiments at higher substrate concentrations for this microsomal preparation just before this experiment. As shown in Fig. 1 *A*, the observed reaction velocities over the range of substrate con-

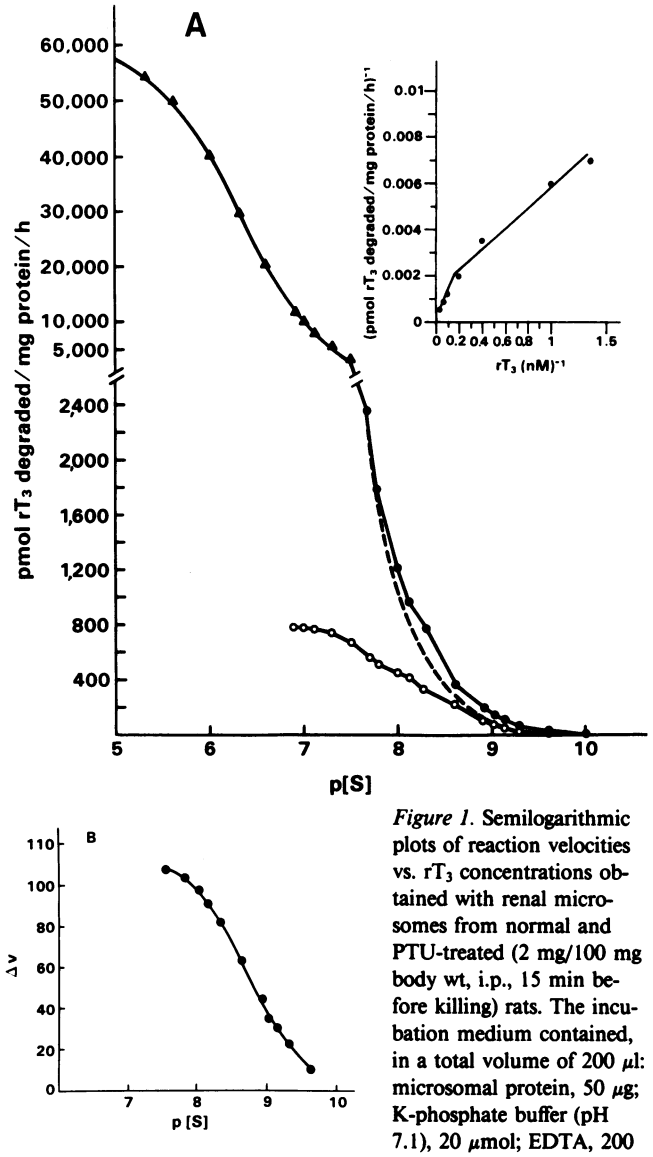


Figure 1. Semilogarithmic plots of reaction velocities vs. rT_3 concentrations obtained with renal microsomes from normal and PTU-treated (2 mg/100 mg body wt, i.p., 15 min before killing) rats. The incubation medium contained, in a total volume of 200 μl: microsomal protein, 50 μg; K-phosphate buffer (pH 7.1), 20 μmol; EDTA, 200 nmol; DTT, 1 μmol and varying amounts of rT_3 (containing 50,000 cpm of ^{125}I-rT_3), as indicated. The incubation was for 1 min and was terminated by the addition of 0.1 ml of 2% BSA, followed immediately by 0.5 ml of 10% TCA. I^- liberated was measured by ion exchange chromatography on Bio-Rad AG50WX8 resin as described (10). (*A*) Activities in normal (●) and PTU-treated (○) microsomes as a function of log 1/[rT_3]. The lower part of *A* shows the activities at nanomolar substrate concentrations on a 25-fold expanded activity scale. Also shown (dashed line) is a theoretical plot of the activities, at low (nanomolar) substrate concentrations, extrapolated from the kinetic parameters observed at high (micromolar) substrate concentrations. The inset shows double reciprocal plots of the activity profile at 0.75–20-nM substrate range. (*B*) Difference between reaction rates observed at low [*S*] and those projected from the kinetic parameters at high [*S*], plotted as a function of log 1/[rT_3]. The reaction rates were calculated as picomoles rT_3 degraded per milligram protein per hour. The data are from one experiment, done in duplicate, and are representative of three similar experiments.

centrations from 0.25 to 10 nM (solid line) were consistently greater than the extrapolated values (dotted line) and showed a sigmoidal profile.[2] The Lineweaver-Burk plots also showed a discontinuity at the 5–10-nM substrate range, as shown in the inset. A difference curve, constructed by plotting the difference between observed and predicted values of enzymatic activity at the low substrate concentrations, also showed a sigmoidal profile (Fig. 1 *B*) suggesting the presence of a low K_m enzymatic activity with a K_m of ~2.5 nM and a V_{max} of ~110 pmol rT_3 degraded/mg protein per h.

This anomalous behavior of the enzyme cannot be explained on the basis of increased fractional binding of substrate to microsomal proteins at low substrate concentrations, as the reaction velocity would be expected to decrease, not increase, because of diminished availability of substrate to the enzyme under such conditions. Moreover, under the conditions used in these experiments, binding of iodothyronine substrate to microsomal proteins was found to be relatively slight (5–10%) and values for percentage binding were within the same range at both micromolar and nanomolar substrate concentrations.[3]

The activity profile of the microsomes from the PTU-treated rats showed the expected sharp decline in activity at substrate concentrations above 100 nM, confirming the findings of Leonard and Rosenberg (3), with relatively less inhibition (35–40%) at lower (0.5–2.5 nM) substrate concentrations. The plot of the PTU-inhibited activity was doubly sigmoid (Fig. 1 *A*) and, while the activity at the higher range of substrate concentrations had a K_m/V_{max} ratio similar to that obtained with normal microsomes at high substrate concentrations, at the low substrate concentration a value for K_m (~2.5 nM) was observed that was similar to that derived from the uninhibited profile at this range, but with a lower V_{max}. While the inhibition pattern at high substrate concentrations is as expected from the well known uncompetitive character of PTU inhibition (3, 11, 12) of the renal and hepatic enzyme, the inhibition profile at low substrate concentrations, characterized by lesser PTU-sensitivity and an unaltered K_m, indicates either some unusual kinetics of a PTU-modified enzyme at low substrate concentrations or the presence of a separate low K_m enzyme with a different mechanism of inhibition by PTU.

The values for K_m and V_{max}, as determined from the linear portions of the Lineweaver-Burk plots at low substrate concentrations in presence of 100 μM PTU were 2.5 nM and 16 pmol/mg protein per h for rT_3 and 0.63 nM and 0.58 pmol/mg protein per h for T_4, respectively, at 2.5 mM DTT. The DTT concentration of 2.5 mM was a compromise to achieve a level of enzyme activity that was detectable by the assay procedure used, yet was not high enough to overcome the inhibitory effects of PTU, especially at high substrate concentrations (10–12). The products of the enzymatic reaction were identified by paper chromatography as 3,3'-diiodothyronine and I^-, and T_3 and I^-, in equimolar proportions, from rT_3 and T_4, respectively. The reaction rates were proportional to the amounts of microsomal protein and the enzymatic activity was completely eliminated by heat treatment (80°C for 1 min) or by pronase digestion of the microsomes before incubation. At high substrate concentrations (range, 0.1 to 10 μM), in the absence of PTU, the kinetic parameters for T_4 and rT_3, as determined by Lineweaver-Burk plots, were: K_m (rT_3), 0.5 μM; K_m (T_4), 5 μM; V_{max} (rT_3), 60 nmol; and V_{max} (T_4), 2.1 nmol/mg protein per h, respectively, in reasonable agreement with published values for kidney microsomes (3); these activities were completely inhibited at 100 μM PTU.

The doubly sigmoid substrate concentration curves, observed in both normal and PTU-treated microsomes (Fig. 1, *A* and *B*), indicate the presence in renal microsomes of either (*a*) a single enzyme with negatively cooperative ligand binding, or (*b*) two enzymes with different K_m values acting on the same substrate (13). This question was then addressed experimentally by inhibition and activation analyses of the enzymatic activities at micromolar and nanomolar substrate concentrations.

Inhibition analysis. PTU inhibited the renal microsomal enzyme, when assayed at 0.5 nM rT_3, by 30% at 100 μM; this was considerably greater than one would expect from the inhibition constant (K_i) (1 μM) reported, and experimentally confirmed, for the uncompetitive inhibition of the high K_m enzyme. As shown in Fig. 2, the inhibition was characterized by partial inhibition kinetics with ~90% inhibition of the total activity at 1 mM PTU, and very little further inhibition at higher concentrations of the inhibitor. The data suggest saturation of a separate inhibitor-binding site. The residual activity in presence of excess of inhibitor was near the limits of experimental detection and was not further analyzed kinetically. In contrast to PTU, iopanoate and dicoumarol inhibited ~65% of the activity at 10 μM.[4] The residual activity, however, was more resistant to these inhibitors, requiring ~2.5 mM

[2] *Reproducibility of data.* For the points in Fig. 1 *A* at which the substrate concentrations were <1 μM, the observed values for rT_3 degradation ranged between 11.7 and 34% of the total and the absolute differences between duplicate determinations were usually <1 and in no case >2.8; the deviations of the duplicates from the means averaged 4%. For the 1-, 2.5-, and 5-μM substrate concentrations, the mean percentages of rT_3 degraded were 8.1, 4.2, and 2.2%, and the duplicates differed by 1.0, 0.4, and 0.6, respectively.

[3] The binding studies were performed by incubation of renal microsomes (50 μg protein) with 0.5 nM and 5 μM labeled rT_3 or T_4 at 4°C for 15 min in 200 μl of 0.1 M potassium phosphate buffer (pH 7.0) followed by ultrafiltration through Millipore HAMK filters (Millipore Corp., Bedford, MA), with analysis of the radioactivity in both the filter and the filtrate. Nonspecific binding was determined after incubation in presence of a 100-fold excess of nonlabeled rT_3 or T_4 and was between 2 and 3% of total radioactivity. Under these conditions the specific binding for rT_3 and T_4 was 8 and 6 fmol at the low and 17 and 11 pmol at the high substrate concentrations, respectively.

[4] This degree of inhibition is close to what one would expect from 10 μM iopanoate (K_i = 9.2 μM) if it were inhibiting only that component of the enzymatic activity attributable to the high K_m enzyme as determined by extrapolation (e.g., as in Fig. 1 *A*).

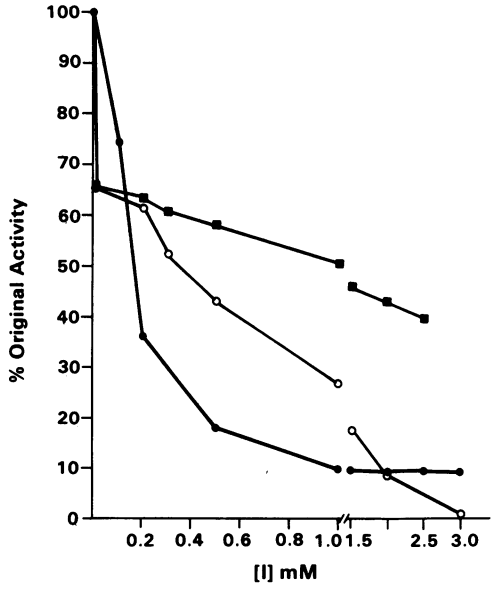
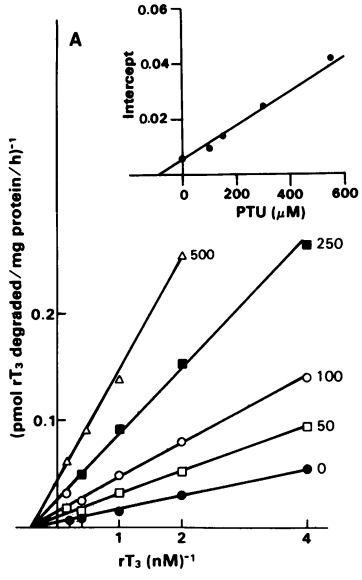


Figure 2. Effects of various concentrations of PTU (●), iopanoate (○), and dicoumarol (■) on microsomal 5'-deiodination of rT_3. The abscissa refers to millimolar concentrations of the inhibitor. The microsomes were incubated for 3 min in absence or in presence of indicated concentrations of specified inhibitors under conditions as described in Fig. 1. rT_3 was 0.5 nM and DTT at 5 mM. The values are from one of three closely agreeing experiments.

iopanoate for complete inhibition, while dicoumarol was much less active (40% inhibition at 2.5 mM). The results indicate the presence of a separate enzyme with different sensitivity to these inhibitors.

Lineweaver-Burk plots at nonsaturating PTU concentrations (Fig. 3 *A*) showed noncompetitive, in contradistinction to uncompetitive, kinetics over the range of lower rT_3 concentrations (0.25–2.5 nM). A replot of the vertical axis intercepts against the inhibitor concentrations was linear (Fig. 3 *A*, inset) and revealed a K_i of ~100 μM, compared with a K_i of 1 μM reported for the uncompetitive inhibition by PTU at high substrate concentrations (14). The inhibition by PTU was competitive with DTT (Fig. 3 *B*), with considerable release of the inhibition at high DTT concentrations. For example, at 550 μM PTU, although the inhibition was ~87% at 2.5 mM DTT, it was only ~56% at 20 mM DTT. A relative insensitivity was also observed with iopanoic acid, a potent inhibitor of microsomal deiodination of iodothyronines at high substrate concentrations (15). The inhibition by iopanoate was competitive with rT_3 (Fig. 4), as is the case with the high K_m enzyme, but the observed K_i (0.9 mM) was over 100-fold greater than that reported for the high K_m enzyme (16). Similar inhibitory patterns [K_i (PTU), 100 μM; K_i (iopanoate), 1.38 mM] were also obtained with the 5'-deiodination of T_4, when assayed at nanomolar substrate concentrations, and the similarity of the K_i's suggested that the outer ring deiodination of both substrates



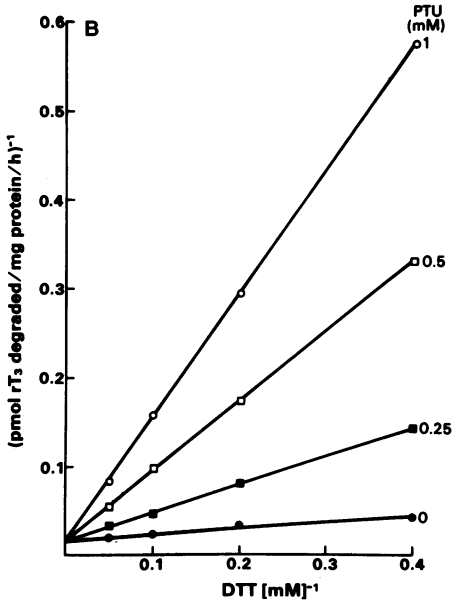


Figure 3. Double reciprocal plots of PTU inhibition of rT_3-deiodinase activity with (*A*) rT_3 as a variable substrate with DTT at 5 mM, and (*B*) DTT as a variable substrate with rT_3 at 0.5 nM. Incubation was for 3 min. The PTU concentrations chosen were nonsaturating (compare Fig. 2), and, in addition to the indicated concentrations, all incubations were performed at a basal PTU level of 50 μM. An intercept replot (*A*, inset) was linear giving a K_i of ~100 μM for the inhibition. The data are from a single representative of three closely agreeing experiments.

at these concentrations was mediated by the same enzyme. The renal enzyme, assayed at low substrate concentrations, thus differs from the type II, low K_m pituitary and brain

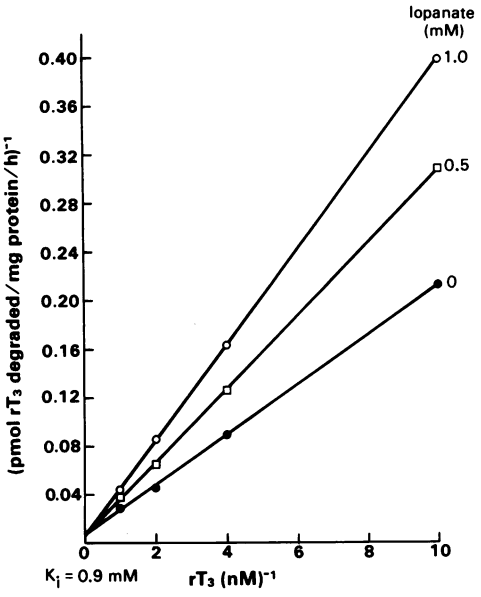


Figure 4. Competitive inhibition of rT_3 5'-deiodinase by iopanoic acid at nanomolar substrate concentrations. Incubation was at 50 μM PTU and 5 mM DTT. Incubation time was 3 min. A K_i of 0.9 mM was calculated for the inhibition. The data are from one of three similar experiments where the values agreed within 10% of each other.

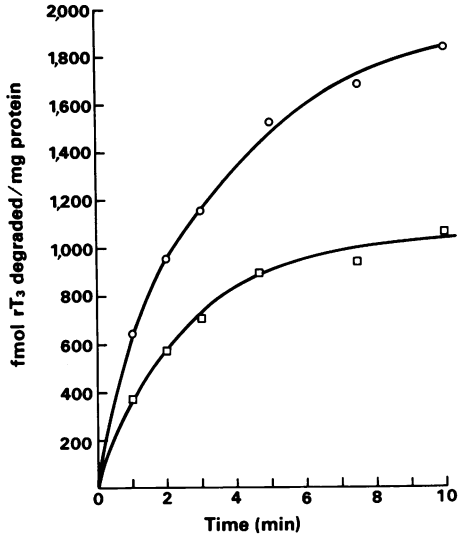


Figure 5. Progress curves of microsomal rT_3 5'-monodeiodinase in 50 μM (○) and 150 μM PTU (□) at 0.5 nM rT_3. The microsomal preparations and the incubation conditions were as described in Fig. 1. The data are from one of two experiments in close agreement.

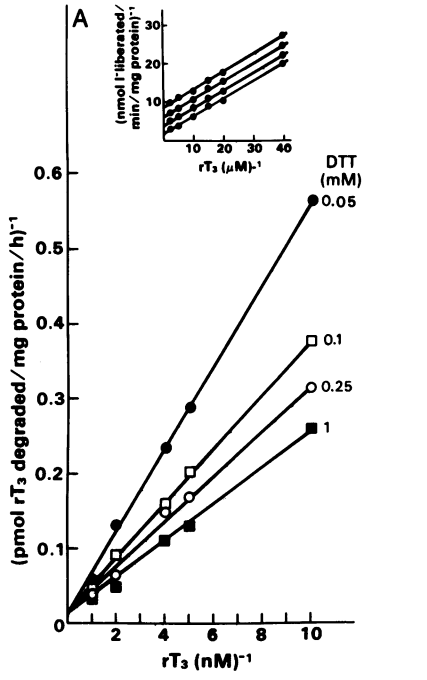
enzymes in being moderately, if incompletely, PTU-sensitive and this may account for the failure of Silva et al. (7) to detect a low K_m enzyme by inhibition analysis alone.

Progress curves. Fig. 5 shows the progress curves of the enzymatic activity at 0.5 nM rT_3 in the presence of 50 and 150 μM PTU. The profiles resembled that reported for the high K_m enzyme (3), composed of an initial rapid phase during the first minute, followed by a slower phase of enzymatic activity. The activity at 150 μM PTU was ~40% inhibited from the levels obtained at 50 μM PTU. This inhibition, however, was observed in both the early and later phases of the progress curve, in contrast to the high K_m progress curve, where the rapid early phase, presumably representing the first catalytic cycle, has been reported to be insensitive to PTU (3). These findings indicate that the 5'-deiodination of rT_3 at nanomolar concentrations is steady and sustained during the entire incubation period and that the relative PTU-insensitivity cannot be attributed to the enzyme activity during the brief (1 min) period of PTU-insensitive catalytic activity.

Reaction mechanisms. Lineweaver-Burk plots of reaction velocities vs. rT_3 concentrations in the nanomolar range at fixed DTT concentrations and in presence of 50 μM PTU generated a series of lines that intersected at the velocity axis (Fig. 6 *A*), in sharp contrast to the parallel lines observed at micromolar substrate concentrations (inset). A similar plot of reaction velocities vs. DTT concentrations at fixed rT_3 concentrations yielded a series of lines intersecting in the second quadrant above the $1/S$ axis (Fig. 6 *B*) and a secondary plot (inset) of the slopes of these lines against $1/rT_3$ yielded a straight line passing through the origin (intercept zero). The data suggest an ordered sequential mechanism for the deiodinase reaction and would seem to indicate the formation of a ternary complex in which rT_3 is the second substrate (17, 18), in contrast to the well documented ping-pong mechanism at high substrate concentrations.

Thermal activation profiles. The effects of temperature on the V_{max} of the enzymatic reaction, when measured at low (nanomolar) substrate concentrations in presence of PTU, also differed significantly from those observed at higher (micromolar) substrate concentrations in absence of PTU. As shown in Fig. 7, while the Arrhenius plots of both activities showed discontinuities (T_d) at ~20°C, the display was concave downwards when measured at a low substrate concentration (20 nM) with activation energies of 5.98 and 11.8 kcal/mol above and below the T_d, respectively. This was in sharp contrast to the profile obtained with 5 μM rT_3, which was concave upwards with activation energies of 2.14 and 7.5 kcal/mol, respectively, above and below the T_d.

Activation energies are regarded as intrinsic characteristics of enzymes rather than substrates (19), and the different thermal activation profiles observed at high and low substrate concentrations can be taken as indirect evidence for two separate enzymes operating at these two substrate levels. It should be noted, however, that the reaction velocities at the low substrate level were measured in presence of PTU and the possibility that the activation profile seen at this substrate concentration was that of a PTU-modified form of a single



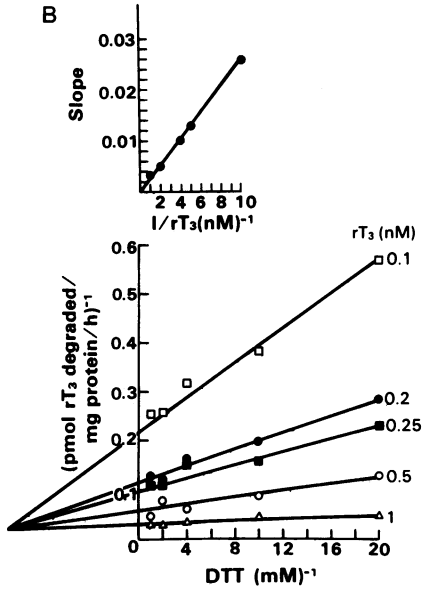


Figure 6. Renal microsomal rT_3 5'-deiodinase activities at nanomolar substrate concentrations with (*A*) DTT and (*B*) rT_3 as fixed substrates. Incubation time was 3 min. In *A*, activity patterns observed at micromolar rT_3 concentrations have been included for comparison. In *B*, a slope replot (inset) was linear passing through the origin. All incubations were performed in 50 μM PTU. The data are from a representative of three closely agreeing experiments. The values agreed to within 10% of each other.

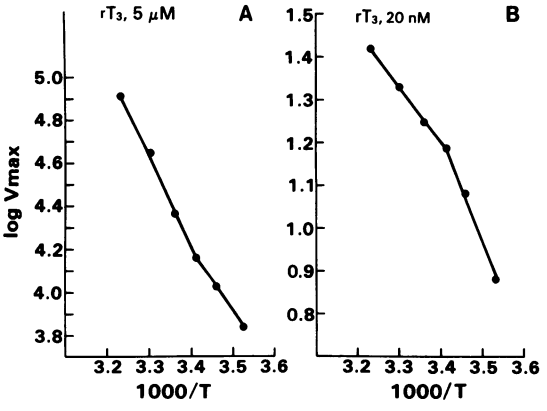


Figure 7. Temperature activation profiles of microsomal rT_3 5'-monodeiodination at micromolar and nanomolar substrate concentrations. Microsomes (50 μg protein) were incubated with 5 μM (*A*) or 20 nM (*B*) rT_3 in presence of 5 mM DTT at the indicated temperatures for 3 min, and the rates of deiodination were measured by methods described in Fig. 1. In *B* the incubation medium also contained 50 μM PTU. The reaction velocities were calculated as picomoles rT_3 degraded per milligram protein per hour. The data are from a representative of three closely agreeing experiments.

enzyme cannot be entirely excluded. The linearity of the Arrhenius plot within the temperature ranges under observation would, however, suggest that the overall velocity is controlled by a single rate limiting step throughout the temperature range (20) and that its thermal activation coefficient is unaffected by interaction with PTU.

Relative substrate preference at high and low [*S*]. Table I shows the 5'-deiodinase activities of rat kidney microsomes with 0.5 nM rT_3 and T_4 in presence of DTT and DHL. In the presence of 5 mM DTT and DHL, while the rT_3 deiodinase activities were only slightly above the values calculated from the kinetic parameters derived from experimental values observed with high substrate concentrations, the T_4 deiodinase activities were about fivefold higher. The preferred substrate for the deiodinating reaction at low substrate concentrations thus appears to be T_4 rather than rT_3. This is also borne out

Table I. 5'-Monodeiodination of T_4 and rT_3 (0.5 nM) by Kidney Microsomes: Effects of Various Thiols

	5'-Monodeiodinase activity (*pmol substrate degraded/mg protein per h*)	
Thiol	T_4	rT_3
DTT	1.1±0.15 (0.21)	93.4±7.5 (60)
DHL	1.4±0.18 (0.21)	104.8±9.7 (60)

Each figure represents mean±SE of data from six experiments in which rT_3 was substrate and four in which T_4 was substrate, each done in duplicate. Assay conditions were as described in the text. The figures in parentheses represent theoretical values calculated from the following kinetic constants (see text): K_m (rT_3), 0.5 μM; K_m (T_4), 5 μM; V_{max} (rT_3), 60; and V_{max} (T_4), 2.1 nmol/mg protein per h.

by the lower K_m for T_4 compared with rT_3 observed at low substrate concentrations (see above) and is a reversal of the relative substrate preference seen at high substrate concentrations.

Thiol activation profiles. The thiol activation of enzymatic activity at nanomolar substrate concentrations was further investigated over a wide range of concentrations of glutathione (GSH), DTT, and DHL, with results as shown in Fig. 8. The previously reported 10-fold greater potency of DHL than of DTT (14) in stimulating deiodinating activity at high substrate concentrations was also found at nanomolar concentrations of substrate. In addition, while the enzyme, in agreement with published data (14), was almost totally unresponsive to 1–5 mM GSH at high substrate levels, it responded to these concentrations of GSH when rT_3 was 0.5 nM, at ~20–25% of the level with DTT. Although the nature of this differential GSH-responsiveness at high and low substrate concentrations is unclear, a possible explanation may be that rat kidney microsomes may contain an operationally distinct GSH-sensitive low K_m enzyme.

A summary of the kinetic parameters of the enzymatic activity at high and low substrate concentrations is presented in Table II.

Discussion

When assayed at low (nanomolar) substrate concentrations, rat kidney microsomal iodothyronine 5'-deiodinase displays some kinetic characteristics that differ significantly from those observed at high (micromolar) substrate concentrations. Some of these differences, notably the relative PTU insensitivity and a sequential reaction mechanism observed at low (nanomolar) substrate concentrations as opposed to the high PTU-sensitivity and the two-step transfer (ping-pong) mechanism, which have been observed at high (micromolar) substrate levels, are reminiscent of the low and high K_m enzymes described in the cerebral cortex (4), pituitary (5), and brown fat (6), and would suggest the presence also of a distinct low K_m enzyme in kidney microsomes. There are, however, several considerations, as discussed below, that must be critically evaluated before such an interpretation can be made.

Inhibition kinetics. (*a*) PTU inhibition of the enzyme at micromolar substrate concentrations is characterized by parallel upward displacements of double reciprocal plots (3). From the kinetic expression of such uncompetitive inhibitions (21, 22): $v = V_{max}/[(K_m/S) + (1 + i/K_i)]$, where i and K_i refer to the inhibitor concentration and the inhibitor constant, respectively, it is clear that inhibition at any fixed concentration of PTU would become progressively lower as substrate concentrations are lowered because the i/K_i term in the denominator becomes small in relation to K_m/S. For example, if K_i (PTU) = 1 μM, K_m (rT_3) = 0.5 μM, and $V_{max}(rT_3)$ = 60 nmol/mg protein per h, although the activity at 0.5 μM rT_3 will be ~85% inhibited at 10 μM PTU, the activity at 0.5 nM rT_3 will be only 1% inhibited (and only 50% inhibited at 1 mM PTU). Conclusions regarding enzyme identification based solely on PTU inhibition are therefore dangerous since even a single enzyme's susceptibility to inhibition varies with experimental conditions (e.g., substrate concentration). (*b*) In most published studies, particularly those dealing with the brain and pituitary deiodinases (4, 5), high DTT concentrations (e.g., 20–100 mM) have been used which might be expected to cause a substantial lessening of PTU inhibition since PTU and DTT are competitive (Fig. 3 *B*). (*c*) Furthermore, under conditions where PTU is inhibitory, the inhibited enzyme would display much lower K_m's and V_{max}'s (depending on PTU concentrations) because of the uncompetitive nature of the inhibition with respect to the iodothyronine substrate (uncompetitive inhibition being characterized by a proportional decrease of K_m and V_{max} with K_m/V_{max} remaining constant). A critical analysis of the kinetic data is therefore imperative in ascertaining whether what is being measured is the residual activity of the high K_m enzyme at low substrate concentrations in an environment of high

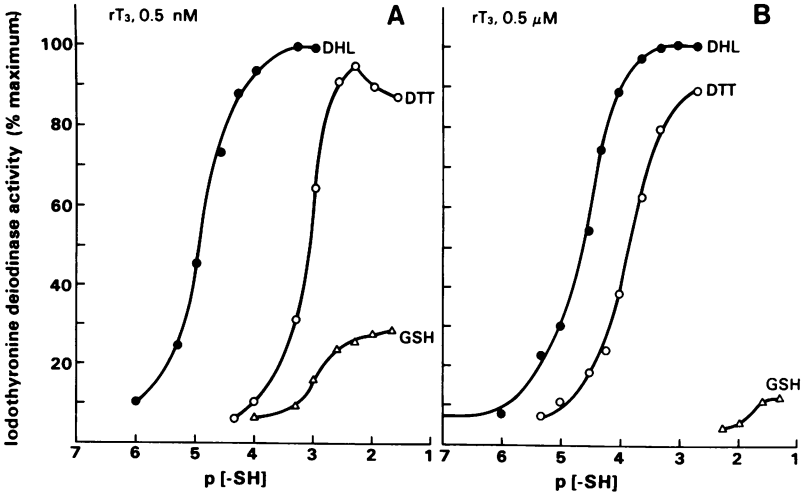


Figure 8. Thiol activation of 5'-monodeiodination of rT_3 by renal microsomes. The incubation conditions were as described in Fig. 1, except that the incubation time was 3 min. Each point is the mean of three determinations, each done in duplicate, from three different microsomal preparations.

Table II. Kinetic Characteristics of Renal Microsomal Iodothyronine 5'-Deiodinase at Low (Nanomolar) and High (Micromolar) Substrate Concentrations

	Low [S]	High [S]
	0.1–10 nM	*0.1–10 μM*
Response to GSH (1–5 mM)	++	0
Inhibition by		
PTU	K_i = 100 μM (Noncompetitive)	K_i = 1 μM (Uncompetitive)
Iopanoate	K_i = 0.9 mM* (Competitive)	K_i = 9.2 μM (Competitive)
Dicoumarol	Minimal*	K_i = 7.5 μM (Competitive)
Mechanism	Sequential (ordered)	Ping-pong
K_m		
rT_3	2.5 nM‡	0.5 μM
T_4	0.63 nM‡	5 μM
V_{max}§		
rT_3	16 pmol‡	60 nmol
T_4	0.58 pmol‡	2.1 nmol
Temperature activation curve	Concave downwards	Concave upwards

* Assayed at 50 μM PTU and 5 mM DTT; ‡ Assayed at 100 μM PTU and 2.5 mM DDT; § mg protein^{-1} h^{-1}.

PTU and DTT, or the activity of a distinctly different low K_m enzyme.

Several distinctive features emerge from analysis of the kinetic data obtained with the kidney enzyme at low substrate concentrations: (*a*) Although the rT_3-deiodinating activity at low [*S*] differed only modestly from that projected from the K_m and V_{max} obtained at high substrate concentrations, T_4-deiodinating activity at low [*S*] was appreciably higher than that extrapolated from the kinetic parameters derived from studies at high [*S*] and, in contrast to the observations at high [*S*], the enzyme showed a much lower K_m for T_4 than for rT_3. Such a reversal of substrate preference for T_4 than for rT_3 has also been reported with the type II enzyme of the brain and pituitary; but unlike the brain and pituitary type II enzyme, where the V_{max}/K_m ratio for T_4 was high and where the rT_3 deiodination was half-maximally inhibited by very low (2 nM) concentrations of T_4 (5), the kidney enzyme at low substrate concentrations showed a much smaller V_{max} for T_4 and required much higher concentrations of T_4 (200 nM) for half-maximal inhibition of rT_3 deiodination (data not shown). The reason for this limited ability of T_4 to suppress rT_3 deiodination, despite the lower K_m for T_4 than for rT_3, is not entirely clear. It should be noted, however, that in a two-substrate sequential mechanism, the K_i of an alternate substrate (e.g., T_4 in respect to rT_3) is not a true dissociation constant, but is a steady-state constant (23) that could assume a high value when the rate of degradation of the ternary complex formed by the competitive inhibitor is slow, as appears to be the case with T_4 relative to rT_3 in the kidney microsomes. (*b*) At 5 mM DTT, 1 mM PTU surprisingly inhibited the enzyme at low [*S*] by >90% (Fig. 2), a level of inhibition that was considerably higher than that calculated from the kinetic parameters at high [*S*]; this suggests the presence of a separate PTU-sensitive, low K_m enzyme. The renal low K_m enzyme thus appears to differ from the type II enzyme described in the cerebral cortex and pituitary (4, 5) in being moderately sensitive to inhibition by PTU. (*c*) The PTU inhibition at low [*S*] was noncompetitive with a K_i considerably larger (100 μM) than the K_i (1 μM) for the uncompetitive inhibition observed at high [*S*]. (*d*) Iopanoate, a competitive inhibitor of rT_3 deiodination (15), was a much weaker inhibitor at low than at high [*S*]. If the high K_m enzyme were the sole renal microsomal outer-ring deiodinase, one would have expected, given the nature of competitive inhibitory kinetics, that a fixed concentration of the inhibitor would be more, rather than less effective at low [*S*]. Thus the observation that the K_i of iopanoate is ~100 times greater at nanomolar than at micromolar [*S*] is additional evidence for the presence of a separate low K_m deiodinase, less sensitive to iopanoate because of its higher affinity for the substrate. Dicoumarol, a potent competitive inhibitor at high [*S*], was a weak inhibitor at low [*S*]; the structural unrelatedness of dicoumarol and iodothyronine raises the possibility that dicoumarol could be a modifier interacting with a separate binding site on the high K_m enzyme alone, perhaps assuming competitive inhibitory kinetics as a K-system effector (24).

To eliminate the activity of the conventional high K_m, PTU-sensitive renal microsomal 5'-iodothyronine deiodinase, we added PTU (50–100 μM) in almost all experiments at low substrate concentrations (except for the experiments in Figs. 1, *A* and *B*, 2, and 8). Actually, the degree of inhibition by PTU of a true high K_m enzyme operating at subnanomolar substrate concentrations is negligible, for reasons explained above. Nevertheless, the possibility that the properties of the low K_m enzyme are really those of a PTU-modified high K_m enzyme needs to be considered. We think it unlikely that this is the case because (*a*) a PTU-modified enzyme would probably not be PTU sensitive, as the low K_m enzyme appears to be; (*b*) PTU modification of the high K_m enzyme would be unlikely to alter iopanoate binding without similarly altering substrate binding; and, (*c*) the difference between predicted and observed reaction rates (Fig. 1, *A* and *B*) at low substrate concentrations were obtained in the absence of PTU.

Reaction mechanisms. The essential feature of the ping-pong mechanism is that the enzyme reacts with one of the substrates to form a Michaelis complex that is followed by the dissociation of the first product leaving a modified enzyme form usually containing a moiety of the first substrate. The second substrate interacts with the modified enzyme only after dissociation of the first product. Based on kinetic data obtained at high substrate concentrations, a model has been proposed

for the hepatic and renal high K_m enzyme in which an oxidized form of the enzyme (possibly a sulfenyl iodide) is first generated during substrate conversion, and reduced iodothyronine product is released; DTT is involved only in the reconversion of the oxidized enzyme to the native form with release of iodide:

$$E + rT_3 \underset{(1)}{\rightarrow} E \cdot rT_3 \underset{(2)}{\rightarrow} T_2 + E_{ox} \cdot I \overset{DTT}{\underset{(3)}{\rightarrow}} E + I^- + DTT_{ox}$$

$$\Big\downarrow (4)\ PTU$$

$$E_{ox} \cdot PTU + I^-.$$

The ping-pong reaction mechanism is usually diagnosed by finding parallel double reciprocal plots of initial rate data and has been experimentally verified for the kidney and liver enzyme in a number of laboratories (10, 11), including our own (3). It must be emphasized, however, as Cleland has done (25, 26), that the parallel initial velocity patterns in ping-pong mechanisms are observed only when the modified enzyme form generated after the release of the first product (in this case $E_{ox} \cdot I$) is kinetically stable during the time period of the reaction. At very low concentrations of the iodothyronine substrate and high concentrations of DTT, this may not be the case, since the rate of formation of the enzyme-substrate complex might conceivably be small as compared with its degradation and rapid equilibrium conditions might not prevail. Furthermore, if $E_{ox} \cdot I$ is the intermediate enzyme form and the mechanism of PTU inhibition is by formation of a mixed disulfide with $E_{ox} \cdot I$ [as envisaged by Leonard and Rosenberg (27) and by Visser et al. (28)], then in presence of excess PTU, the reaction will be diverted towards the formation of the PTU-complex (step 4) and the apparent first-order rate constant for the reaction of enzyme and substrate to give iodide will be nearly zero (ignoring the minute amount of iodide generated during the first catalytic cycle). Raising DTT levels will divert the reaction flux from the dead-end formation of the PTU-complex (step 4) (or the spontaneous hydrolysis of the $E_{ox} \cdot I$ complex) to thiol-dependent production of iodide (step 3) and thus increase the apparent rate of iodide generation (without affecting the true rate of combination between E and S). This would decrease the slope of the reciprocal plot[5] and intersecting rather than parallel lines would be observed on Lineweaver-Burk plots, even if the reaction mechanism is basically ping-pong. This becomes particularly relevant at low substrate concentrations where the PTU-inhibition is small and measurable reaction rates can be obtained even at fairly high concentrations of PTU. Considerable caution must therefore be exercised in interpreting the intersecting Lineweaver-Burk plots at very low iodothyronine concentrations (as observed in the present studies and in those of Larsen and co-workers [4–6]), as evidence for a separate (sequential) kinetic mechanism and, therefore, for a separate enzyme.

Whether the reaction mechanisms observed at high and low substrate concentrations are different or not, other manifestations of the enzymatic activity at low substrate concentrations, notably the differential response to GSH, differential response to both uncompetitive and competitive inhibitors and dissimilar Arrhenius displays are, indeed, suggestive of the presence of a separate low K_m enzyme in rat kidney microsomes. Such a low K_m enzyme could be quantitatively important at physiological concentrations of circulating T_4 and could make a significant contribution to the T_3 production rate. An estimate, based on our experimentally observed renal and hepatic microsomal yields in rats (assuming similar kinetic properties for the enzyme activities in these two organs), indicates that at circulating levels of free T_4 [3×10^{-11} M (29)], ~38 and 80 pmol of T_3/100 g body wt could be generated daily by the high and low K_m enzymes, respectively, in these two organs, thus accounting for approximately half of the total daily production rate of T_3 in rats, estimated by Abrams and Larsen (30) to be ~250 pmol/100 g/body wt d. The findings of Lum et al. (31) that the serum T_3 autoregulatory system in euthyroid humans with low circulating T_4 concentrations is minimally affected by PTU and iopanoate might be explained on the basis of the relative insensitivity of the low K_m enzyme to these two inhibitors. The relative insensitivity to PTU and the partial inhibitory kinetics observed with this inhibitor when enzyme activities are measured at low substrate concentrations may also account for the observations of Silva et al. (6) that, in PTU-treated rats, circulating T_3 concentrations cannot be lowered below about one-third of the normal levels (even with a dosage as high as 1 mg/100 g body wt).

[5] The lack of slope effect in double reciprocal plots of ping-pong mechanisms is due to a constancy of the ratio of free enzyme [E] to the central complex (F), irrespective of the concentration of the second substrate, because of a lack of reversible connection between E and F (23).

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References

1. Fekkes, D., E. Van Overmeeren, G. Hennemann, and T. J. Visser. 1980. Solubilization and partial characterization of rat liver iodothyronine deiodinases. *Biochim. Biophys. Acta.* 613:41–51.

2. Leonard, J. L., and I. N. Rosenberg. 1981. Solubilization of a phospholipid-requiring enzyme, iodothyronine 5'-deiodinase, from rat kidney microsomes. *Biochim. Biophys. Acta.* 659:205–218.

3. Leonard, J. L., and I. N. Rosenberg. 1980. Iodothyronine 5'-deiodinase from rat kidney: substrate specificity and the 5'-deiodination of reverse triiodothyronine. *Endocrinology.* 107:1376–1383.

4. Visser, T. J., J. L. Leonard, M. M. Kaplan, and P. R. Larsen. 1982. Kinetic evidences suggesting two mechanisms for iodothyronine 5'-deiodination in rat cerebral cortex. *Proc. Natl. Acad. Sci. USA.* 79:5080–5084.

5. Visser, T. J., M. M. Kaplan, J. L. Leonard, and P. R. Larsen. 1983. Evidence for two pathways of iodothyronine 5'-deiodination in

rat pituitary that differ in kinetics, propylthiouracil sensitivity, and response to hypothyroidism. *J. Clin. Invest.* 71:992–1002.

6. Leonard, J. L., S. A. Mellen, and P. R. Larsen. 1983. Thyroxine 5'-deiodinase activity in brown adipose tissue. *Endocrinology.* 112:1153–1156.

7. Silva, J. E., J. L. Leonard, F. R. Crantz, and P. R. Larsen. 1982. Evidence for two tissue-specific pathways for in vivo thyroxine 5'-deiodination in the rat. *J. Clin. Invest.* 69:1176–1184.

8. Reed, L. J., M. Koike, M. E. Levitch, and F. R. Leach. 1958. Studies on the nature and reaction of protein-bound lipoic acid. *J. Biol. Chem.* 232:143–158.

9. Silver, M. 1979. Thin layer chromatography of lipoic acid, lipoamide and persulfides. *Methods Enzymol.* 62:135–137.

10. Goswami, A., J. L. Leonard, and I. N. Rosenberg. 1982. Inhibition by coumadin anticoagulants of enzymatic outer ring monodeiodination of iodothyronines. *Biochem. Biophys. Res. Commun.* 104:1231–1238.

11. Chopra, I. J., S. Y. Wu, Y. Nakamura, and D. H. Solomon. 1978. Monodeiodination of 3,5,3'-triiodothyronine and 3,3',5'-triiodothyronine to 3,3'-diiodothyronine in vitro. *Endocrinology.* 102:1099–1106.

12. Visser, T. J. 1979. Mechanism of iodothyronine 5'-deiodinase. *Biochim. Biophys. Acta.* 569:302–308.

13. Dixon, H. B. F., and K. F. Tipton. 1973. Negatively cooperative ligand binding. *Biochem. J.* 133:837–842.

14. Goswami, A., and I. N. Rosenberg. 1983. Stimulation of iodothyronine outer ring monodeiodinase by dihydrolipoamide. *Endocrinology.* 42:1180–1187.

15. Chopra, I. J., D. H. Solomon, U. Chopra, S. Y. Wu, D. A. Fisher and Y. Nakamura. 1978. Pathways of metabolism of thyroid hormones. *Recent Prog. Horm. Res.* 34:521–567.

16. Fekkes, D., G. Hennemann, and T. J. Visser. 1982. One enzyme for the 5'-deiodination of 3,3',5'-triiodothyronine and 3',5'-diiodothyronine in rat liver. *Biochem. Pharmacol.* 31:1705–1709.

17. Dixon, M., and E. C. Webb. 1979. Enzymes. Academic Press, New York. 90–91.

18. Rudolph, F. B., and H. J. Fromm. 1979. Plotting methods for analyzing enzyme rate data. *Methods Enzymol.* 63:138–159.

19. Sizer, I. W. 1943. Effects of temperature on enzyme kinetics. *Advances Enzymol.* 3:35–62.

20. Wong, J. T.-F. 1975. Kinetics of Enzyme Mechanisms. Academic Press, New York. 219–223.

21. Dixon, M., and E. C. Webb. 1979. Enzymes. Academic Press, New York. 342.

22. Cleland, W. W. 1963. The kinetics of enzyme-catalyzed reactions with two or more substrates or products. II. Inhibition: nomenclature and theory. *Biochim. Biophys. Acta.* 67:173–187.

23. Dixon, M., and E. C. Webb. 1979. Enzymes. Academic Press, New York. 105–109.

24. Frieden, C. 1964. Treatment of enzyme kinetic data. 1. The effect of modifiers on the kinetic parameters of single substrate enzymes. *J. Biol. Chem.* 239:3522–3531.

25. Cleland, W. W. 1970. Steady state kinetics. *In* The Enzymes (Student Edition). Vol. II. P. D. Boyer, editor. Academic Press, New York. 1–65.

26. Cleland, W. W. 1963. The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations. *Biochim. Biophys. Acta.* 67:104–137.

27. Leonard, J. L., and I. N. Rosenberg. 1980. Characterization of essential enzyme sulfhydryl groups of thyroxine-5'-deiodinase from rat kidney. *Endocrinology.* 106:444–451.

28. Visser, T. J., D. Fekkes, R. Docter, and G. Hennemann. 1978. Sequential deiodination of thyroxine in rat liver homogenates. *Biochem. J.* 174:221–229.

29. Refetoff, S., N. I. Robin, and V. S. Fang. 1970. Parameters of thyroid function in serum of 16 selected vertebrate species: a study of PBI serum T_4, free T_4, and the pattern of T_4 and T_3 binding to serum proteins. *Endocrinology.* 86:793–805.

30. Abrams, G. M., and P. R. Larsen. 1973. Triiodothyronine and thyroxine in the serum and thyroid glands of iodine-deficient rats. *J. Clin. Invest.* 52:2522–2531.

31. Lum, S. M. C., R. Morris, C. A. Spencer, and J. T. Nicoloff. 1983. Dual peripheral 5'-deiodinase (5'D) systems affecting circulating T_3 levels in man. *65th Annu. Meet. Endocrine Soc.*, 266. (Abstr.)