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Iodothyronine 5'-Deiodinase: Solubilization, Partial Purification, and Characterization of a Thyroxine Deiodinase Extracted from Rat Liver Membranes

Samuel C. Powell
Loyola University Chicago

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IODOTHYRONINE 5'-DEIODINASE

Solubilization, Partial Purification, and Characterization of a

Thyroxine Deiodinase Extracted From Rat Liver Membranes

by

Samuel C. Powell

A Dissertation Submitted to the Faculty of the Graduate School of

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1982

The Soul enters by the throat

-Old Turcoman saying

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VITA

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CHAPTER I

INTRODUCTION

Origin of The Thyroid Hormones

The thyroid gland, an endocrine gland lying across the trachea
lateroinferior to the thyroid cartilage, is the source and primary regul-
ator of serum thyroxine (T4) concentrations (Taurog, 1978). In addition
to thyroxine, its major hormone product, the thyroid gland also produces
small amounts of two other iodinated thyronine molecules: triiodothy-
ronine (T3) and reverse triiodothyronine (rT3) (Chopra et al., 1978).
The production of these hormones by the follicular cells of the thyroid
gland occurs in four steps: 1) elemental iodine is concentrated, 2)
tyrosine residues in the protein thyroglobulin are iodinated, 3) iodi-
nated tyrosines in thyroglobulin are coupled to form iodothyronine, and
4) the hormone product is released from thyroglobulin by enzymatic hydro-
lysis (Freiden and Lipner, 1972). Every animal species found in nature
has specific cells or tissues capable of the first two steps, trapping
and storing elemental iodine in an organic form (Gorbman, 1978). How-
ever, the ability to regulate the formation of iodinated thyronine resi-
dues from the oxidative coupling of two iodinated thyronine residues, and
the ability to regulate the subsequent enzymatic hydrolysis of the iodi-
nated thyronine residues are biochemical phenomena found only in members
of the vertebrate animal kingdom (Gorbman, 1978). Indeed, all adult mem-
bers of vertebrate species studied thus far have follicular thyroid cells
capable of producing the iodinated thyronines (Henderson and Gorbman,
1971).

In addition to the thyroid gland, other extrathyroidal tissues, primarily the liver and kidney have been shown to contribute to the serum concentrations of T3 (Braverman et al., 1970) and reverse T3 (Chopra, 1976). These peripheral tissues contain specific deiodinases which produce T3 and rT3 as a result of the monodeiodination of T4 (Visser et al., 1976a). It has been reported that as much as 80 % of the circulating serum T3 and as much as 97 % of the circulating reverse T3 may be derived from the degradation of T4 in the peripheral tissues (Chopra et al., 1975a; Schimmel and Utiger, 1977). The continued stepwise deiodination of thyroxine in the peripheral tissues also results in the production of diiodinated and monoiodinated thyronines (Chopra et at., 1975a; Smallridge et al., 1979; Pangaro et at., 1980).

The Thyroid Hormones

Thyroxine (T4)

Thyroxine was discovered by Edward Kendall on Christmas Day, 1914, when the first purified crystals of thyroxine (T4) were obtained from an alkaline hydrolysate of bovine thyroid glands (Kendall, 1929). The product he obtained was biologically active and gave a cherry red color when reacted with nitrous acid (Kendall and Osterberger, 1919). Kendall proposed that the substance was an iodinated derivative of tryptophan and he orignally named the compound thyroxindole. Harrington and Barger identified the correct structure (Figure 1) in 1927, and changed the name of the compound to thyroxine (Harrington and Barger, 1927). Interestingly, Kendall originally chose the name thyroxindole rather than the one that

Figure 1. Molecular structure and numbering system for thyroxine (T4).

A. The molecular structure of thyroxine consists of an diiodo-substituted phenol ring attached to a diiodotyrosine by an ether linkage. The oxygen bridge imposes an angle of 120° on the axis of symmetry between the 2 aromatic rings. Despite a steric hindrance by the bulky iodine atoms of the inner ring, molecular orbital calculations indicate that the energy barrier to rotation of the outer ring about its axis of symmetry is sufficiently low to allow rapid rotation at room temperature (Kollman et al., 1973). The chemical formula for T4 is: $C_{15}H_{11}O_4N\ I_4$. Thyroxine has a molecular weight of 776.88 g/mol. Of its total weight, 1.8% is nitrogen and 65.4% is iodine. B. The adopted numbering system for the aromatic rings is as shown. Primed numbers are used to number the carbon atoms of the outer ring. The chemical name for thyroxine is: L-beta-3,5-diiodo-4-(3',5'-diiodo-4'-hydroxyphenoxy)-phenyl-alpha-amino proprionic acid. Thyroxine is classified in Chemical Abstracts as a derivative of the amino acid alanine: alanine,-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl].

A
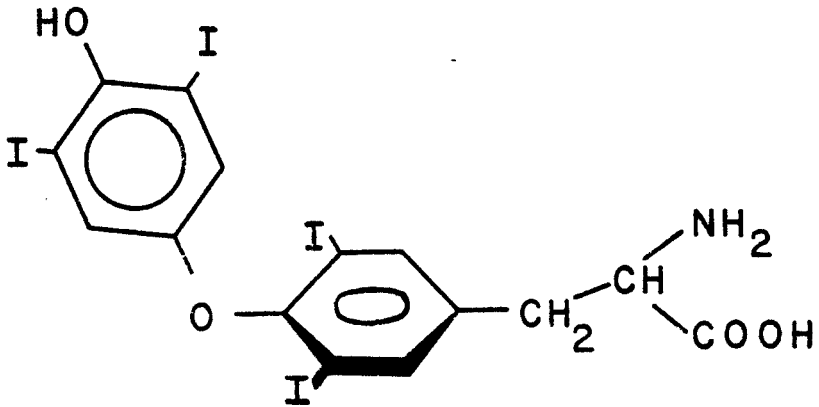
3,3',5,5'- tetraiodothyronine (T4)

B

4'-hydroxyl | outer ring | inner ring | alanine side chain
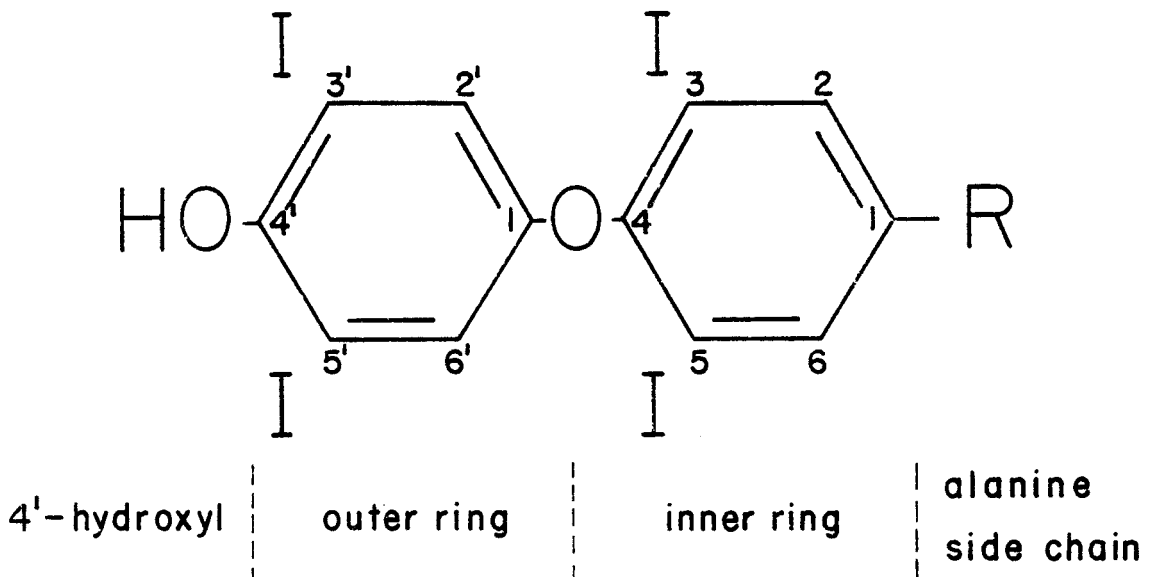
would emphasize the iodine component because he felt that it was the structure of the organic nucleus and not the iodine content that gave the hormone its physiologic activity (Kendal, 1929). This original idea has recently been proven to be true by demonstration of thyroid hormonal activity in halogen-free analogs of T4 (Jorgensen, 1978).

Trevorrow (1939) first proposed the presence of T4 in human serum (Trevorrow, 1939). He found that most of the iodine in plasma coprecipitated with serum proteins and was extractable with organic solvents. The actual identification of plasma T4, however, was first accomplished nine years later with the successful crystallization of T4 from human serum (Taurog and Chaikoff, 1948). More recently, studies using radioimmunoassay procedures have substantiated the existence of circulating T4, and have indicated that it may account for up to 90 % of the total protein-bound plasma iodide (Beckers et al., 1972; Chopra, 1978). Normal human serum concentrations of T4 are in the range of 4.5 to 11.5 μg/100 mL (Chopra et al., 1975a).

3,3',5-Triiodothyronine (T3)

Thyroid hormone and thyroxine (T4) became synonymous terms following the establishment of the structure of thyroxine in 1927, by Harrington and Barger. For many years, thyroxine was considered the only thyroid hormone, and clinicians began an effort to relate its serum concentrations to thyroid disease states. Then, in 1954, Gross and Pitt-Rivers reported the existence of a second iodinated thyronine in normal human serum as well as in the thyroid gland itself (Gross and Pitt-Rivers, 1954). This compound contained 3 iodine atoms and became known

as triiodothyronine (T3) (See Figure 2). This "new" hormone was effective in treating myxedema. Studies in thyroidectomized rats showed that T3 had a more rapid onset of action and was from 3 to 7 times more potent than T4 in stimulating oxygen consumption and in preventing goiter formation. Therefore, these workers proposed that while T4 was the major form in which thyroid hormone was secreted, triiodothyronine (T3) was the more active form within the cells of the target tissues (Gross and Pitt-Rivers, 1954). This theory is still valid and at the present time T3 is considered to be the most physiologically active of the naturally occurring iodothyronines (Ingbar and Braverman, 1975; Latham et al., 1976; Chopra, 1978; Jorgenson, 1978).

Triiodothyronine (T3), like T4 is produced by the thyroid gland, however, the primary source of serum T3 is the enzymatic monodeiodination of T4 to T3 in peripheral tissues (See Figure 3) (Braverman et al., 1970; Chopra, 1978). As a result of both thyroidal and peripheral production, the human serum concentration of T3 is normally in the range of 92 to 190 ng/100 mL (Chopra et al., 1975a). The enzyme responsible for the conversion of T4 to T3 removes a single iodine atom from the 5'-position in the phenol ring of T4 and, therefore, is described as an iodothyronine 5'-deiodinase. The biologic significance of the conversion of T4 to T3 is considered to be that of an activating pathway whereby T4 functions as a prohormone.

3,3',5'-Triiodothyronine (rT3)

The enzymatic conversion of T4 to T3 in peripheral tissues leading to increased plasma T3 concentrations is now well established. In addi-

Figure 2.   Iodothyronines containing 3 iodine atoms per molecule.

The molecular configuration is shown for triiodothyronine (T3) and
reverse T3 (rT3).   The outer ring of T3 has a single iodine substituant.
In comparison, the inner ring of rT3 has a single iodine substituant.   By
convention, the lower number is used in the descriptive term, and a 3
instead of a 5 is used to indicate the position of iodine in the outer
ring of T3 and in the inner ring of rT3.

HO—⟨I⟩—O—⟨I⟩—CH₂—CH(NH₂)COOH

3,3',5-triiodothyronine (T3)

HO—⟨I⟩—O—⟨I⟩—CH₂—CH(NH₂)COOH
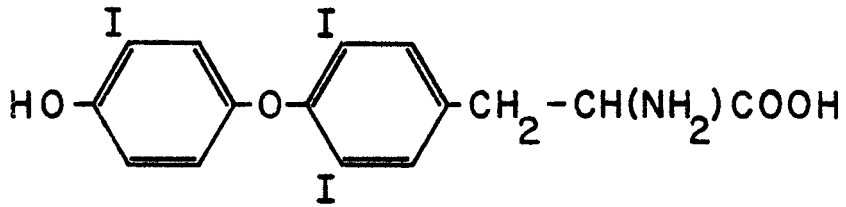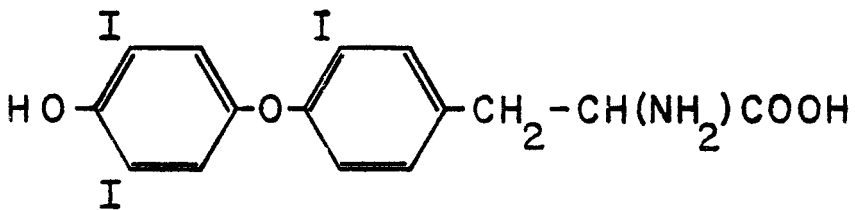
3,3',5'-triiodothyronine (rT3)

Figure 3. Relative significance of thyroidal and extrathyroidal sources
of T3, T4 and reverse T3 (rT3).

An approximation of the amount of hormone excreted daily by the
thyroid gland, and the amount of hormone derived from the peripheral
deiodination of T4 each day is diagrammed based on data from previous
publications (Schimmel and Utiger, 1977; Chopra et al., 1978). Of the
approximate 90 μg of T4 produced each day by the thyroid gland, approxi-
mately 30 μg (33 %) is converted to T3, a slightly greater amount, 43 μg
(48 %) is converted to rT3, and the remaining 17 μg (19 %) is metabolized
by processes other than deiodination. (Figure based on diagram presented
by Schimmel and Utiger, 1977.)

THYROID

produced in thyroid

produced in peripheral tissues from T4

T3

T4

rT3

iodothyronine
5'-deiodinase

iodothyronine
5-deiodinase

T3

T4

rT3

36 μg/day

90 μg/day

46 μg/day

6 μg
30 μg

90 μg

3 μg
43 μg
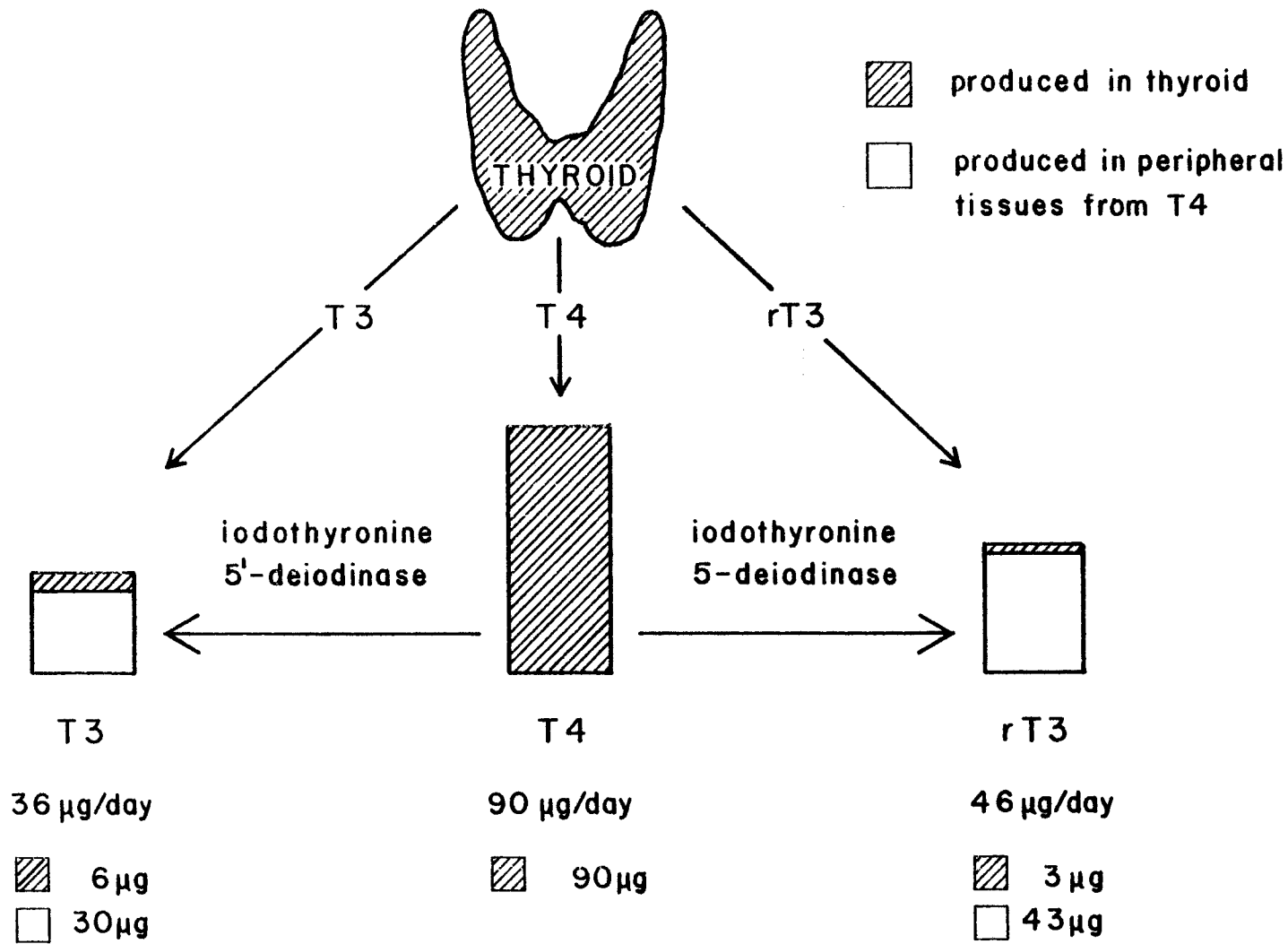
tion, the peripheral tissues also convert a nearly equal amount of T4 to a second triiodothyronine commonly referred to as reverse T3 (Figure 3). Reverse T3 (rT3) (Figure 2), is found in normal serum in a concentration range of 27 to 62 ng/100 mL (Chopra et al., 1975a; Vagenakis et al., 1975). It is currently proposed that a specific monodeiodinase, "iodo-thyronine 5-deiodinase" is responsible for the conversion at T4 to rT3 (Cavalieri et al., 1977; Chopra et al., 1978; Sakurada et al., 1978; Kaplan and Utiger, 1978; Gavin et al., 1980). In contrast to T3, rT3 is calorigenically inactive (Stasilli et al., 1959; Gavin et al., 1977; Chopra, 1978) and the conversion of T4 to rT3 is regarded as an inactiv-ating pathway for T4 metabolism. The iodothyronine 5-deiodinase is responsible for the production of most, if not all, of the circulating serum rT3 (Schimmel and Utiger, 1977; Gavin et al., 1977).

In addition to being calorigenically inactive, rT3 has also been shown to have a negative effect on the calorigenic activity of both T4 and T3 (Pittman and Barker, 1959, Pittman et al., 1960) and has actually been used to treat hyperthyroidism (Benua et al., 1959). Reverse T3, however, may not be antagonistic to T4 and T3 in all of its effects. For example, at pharmacologic doses, the effect of rT3 is as potent or more potent than T3 in the inhibition of phosphodiesterase activity in rat calvaria (Marcus et at., 1975), and in stimulating erythropoiesis in mouse bone marrow cells (Golde et al., 1976). At normal concentrations, however, rT3 appears to be more antagonistic than additive to the overall thyroid effect (Chopra, 1978) and, therefore, is generally considered to be a negative effector of thyroid hormone activity.

Other Thyroxine Metabolites

The thyroxine metabolites containing less than 3 iodine atoms per molecule (See Figure 4) are considered to be of only minor physiological importance. These compounds have biological half-lives of less than one hour, and normal human serum concentrations are in the range of from 0.8 to 17 ng/dL (Chopra et al., 1972; Burman et al., 1977; Smallridge et al., 1979; Meinhold and Schurnbrand, 1978; Pangaro et al., 1980). These partially iodinated thyronines are thought to be the result of continued exposure of T3 and rT3 along with their metabolites to the peripheral deiodinase enzymes. For example, it has been shown that thyroidectomized rats will metabolize radiolabelled T3 or rT3 in vivo to 3,3'-T2 (Roche et al., 1956; Flock et al., 1960). The in vitro conversion of T3 and rT3 to 3,3'-T2 in rat liver homogenates has also been demonstrated (Chopra, 1976). These deiodinases are not to be confused with those normally present in the thyroid and concerned with iodide conservation. All of the partially iodinated thyronines shown in Figure 4 have been detected in normal human serum using radioimmunoassay procedures suggesting, therefore, that thyroxine can be completely deiodinated in the peripheral tissues. The iodine released during peripheral deiodination is conserved by the body (Surks and Oppenheimer, 1969).

The Hormonal Status of Thyroxine

The physiological importance of T4 as an intrinsically active thyroid hormone has recently been questioned (Braverman et al., 1970; Schwartz et al., 1971; Oppenheimer et al., 1972). Increasing evidence indicates that in the euthyroid state most, if not all, of the metabolic
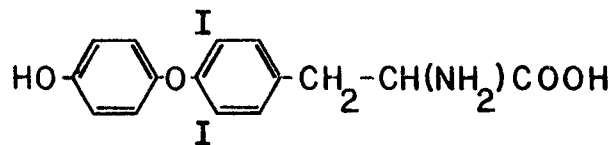
3,5-diiodothyronine (3,5-T2)
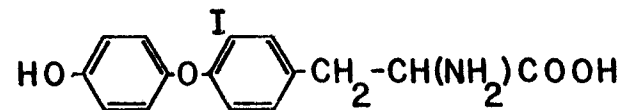
3-monoiodothyronine (3-T1)
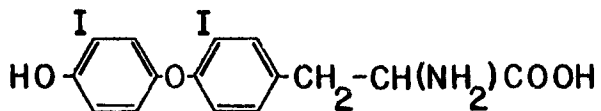
3,3'-diiodothyronine (3,3'-T2)
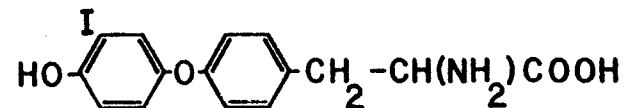
3'-monoiodothyronine (3'-T1)
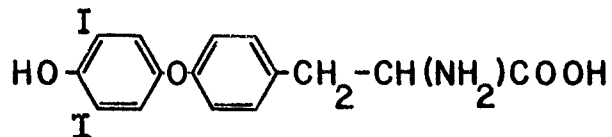
3',5'-diiodothyronine (3',5'-T2)
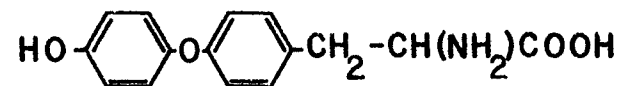
thyronine (T∅)

Figure 4. Structural formula for iodothyronines containing 2 iodine atoms or less per molecule.

effect of the thyroid hormones can be attributed to T3. During certain disease states, when the serum ratio of T3 to T4 is decreased, the contribution of T4 to the metabolic effect may increase, However, in the normal healthy adult the contribution of T4 to the overall metabolic effect of the thyroid hormones is estimated to be 15 % or less, and T4 is thought to serve the body primarily as a reservoir of prohormone for T3 (Ingbar and Braverman, 1975; Chopra, 1978).

One way in which the relative contributions of T4 and T3 to the biologic effect of the thyroid hormones has been estimated is on the basis of daily turnover rates and the relative potency of T3 and T4. Data now available indicate that the calorigenic activity of T3 is from 2 to 4 times greater than that of T4, and that from 1/3 to 1/2 of the T4 metabolized each day is converted to T3 in the peripheral tissues (Brown et al., 1974; Braverman et al., 1970). Based on this data, Brown and colleagues estimated that 65 % of the physiological effect of the thyroid hormones produced each day is due to T3 while 35 % is due to T4 (Brown et al., 1974). This estimate was later modified to reflect the fact that rT3, which has less than 5 % of the calorigenic activity of T4 (Pittman et al., 1962; Stasilli et al., 1959), is also a major product of the peripheral metabolism of T4. Taking into account the metabolism of T4 to rT3, the estimated contribution of T4 to the biologic effect of thyroid hormones was reduced from 35 % to about 14 % (Chopra, 1978). In addition, it was pointed out that the original estimate was based on a daily turnover rate of 110 μg T4/day. If the more commonly observed value of 90 μg T4/day is used, the final estimate of the activity attributable directly to intact T4 would decrease nearly to zero (Chopra, 1978).

A second approach to determining the relative contribution of T4 and T3 to the metabolic effect of the thyroid hormones has been estimating the cross-reactivity of T4 with T3 at nuclear binding sites. This approach is based on the assumption that the binding of hormone to nuclear binding proteins (NBP) is responsible for the initiation of hormonal action, and that the contribution of T4 and T3 to total hormonal activity depends on the mass of T4 and T3 bound to the NBP sites (Oppenheimer and Surks, 1975; Mariash and Oppenheimer, 1980). Perhaps the best estimates of the binding capacities of nuclear sites have been generated under in vivo conditions (Latham et al., 1976). Binding studies by Oppenheimer and colleagues utilized in vivo isotopic studies to determine binding capacities of T3 and T4 to rat hepatocytes (Oppenheimer et al., 1974). Their results indicated that T4 binds to the same NBP sites as T3, but with an association constant approximately 1/20 that of T3. The actual content of T3 and T4 bound specifically to the rat liver NBP was calculated to be 20.3×10^{-13} and 2.83×10^{-13} mol/g liver tissue respectively (Oppenheimer et al., 1974). This data predicts that the biological effect of T3 exceeds the intrinsic contribution of T4 by a factor of about 7, or that approximately 12 % of the total metabolic activity of the thyroid hormones is due to T4 (Oppenheimer and Surks, 1975). Similar results were predicted for the relative potency of the thyroid hormones in man (Oppenheimer et al., 1974).

In another set of in vivo experiments propylthiouracil (PTU), a potent inhibitor of the iodothyronine 5'-deiodinase, was given to hypothyroid patients receiving supplemental thyroxine. The administration of PTU did not cause a significant change in serum T4 levels, however, it

did result in decreased serum T3 levels and a modest increase in serum thyroid-stimulating hormone (TSH) levels (Geffner et al., 1975). Propyl-thiouracil has also been shown to be associated with anti-T4 effects in the rat (Oppenheimer et al., 1972). Results from the studies with PTU were interpreted to indicate that T3 may be the sole biologically active thyroid hormone (Oppenheimer et al., 1972; Chopra, 1978).

Two theories have been proposed to explain why T3 has a greater biological potency than T4. The first theory suggests that the iodine in the 5'-position of T4 creates steric hindrance to binding of T4 at receptor sites (Oppenheimer and Surks, 1975). Binding to the functional receptor would be more difficult for T4, therefore, than for T3, which has only one iodine atom in the phenolic ring. The second theory is based on the fact that at physiological pH, the 4'-hydroxyl group of T4 is dissociated while the 4'-hydroxyl group of T3 is not (Latham et al., 1976). The presence of a single iodine in the phenol ring of T3 peturbs the pKa (-log of the dissociation constant) of the 4'-hydroxyl group from 10.1 as in tyrosine to 8.40, and the second iodine in the phenol ring of T4 peturbs the pKa of the 4'-hydroxyl group to approximately 6.46 (Smith, 1970). It is suggested, therefore, that the presence of a 4'-hydroxyl group at physiological pH gives T3 a greater binding affinity than T4 for the functional receptor proteins of the target cell (Latham et al., 1976). This theory has been supported by the demonstration that T3 binds optimally to rat liver NBP at pH 7.6 and that T4 binds optimally to rat liver NBP at pH 6.0 (Latham et al., 1976; Eberhardt et al., 1979). It has also been shown that above pH 8.2, the binding affinity of T3 for rat liver NBP decreases to a point equal to or less than the binding affinity

of T4 (Latham et al., 1976).

peripheral Metabolism of Thyroid Hormones

Iodothyronines undergo transformations in the peripheral tissues by three major routes: conjugation, deiodination, and degradation. Conjugation occurs in hepatic tissues and results in the formation of sulfate ester and glucuronide conjugates which are excreted in the bile (Bhagavan, 1974; White et al., 1978). There is normally a high tubular resorption capacity for free T3 and T4, and very little if any thyroid hormone is excreted in the urine (Adlkofer et al., 1980). Deiodination occurs by a specific set of enzymes which act in a stepwise manner to strip iodine from either the inner or outer ring of the iodothyronines. These enzymes are located in the liver and kidney as well as many other peripheral tissues (Chopra, 1977a). Degradation occurs by several enzymatic pathways: An hepatic aminotransferase is active in the deamination of both iodotyrosines and iodothyronines and results in the formation of pyruvic analogs of the thyroid hormones (White et al., 1978). The formation of acetic acid analogs occurs in the kidneys by a two-step process which includes an initial deamination and a subsequent decarboxylation step (Braverman et al., 1970). The liver catalyzes an O-methylation of the acetic acid analogs utilizing S-adenosylmethionine (SAM), and the liver and kidney both catalyze the rupture of the diphenyl ether bridge, the ortho hydroxylation of the phenol rings, and the degradation of the alanine side chain (White et al., 1978). The three major routes are not mutually exclusive and pyruvic acid and acetic analogues of T4, T3 and rT3 are found in normal human serum (Chopra et al., 1978).

Deiodination is the primary step in the catabolic pathway for thyroxine. It is estimated that of the total amount of T4 removed from the plasma pool each day, 70 % to 80 % undergoes peripheral deiodination, another 15 % to 20 % is conjugated and excreted in the bile, and the remaining 5 % to 10 % is deaminated or decarboxylated (Bhagavan, 1974; Ingbar and Braverman, 1975; White et al., 1978).

The deiodination of T4 in the peripheral tissues is of uppermost biological importance. In addition to being the major catabolic route of thyroxine metabolism, the bidirectional deiodination of T4 (See Figure 3) is responsible for the production of approximately 80 % of the plasma T3 and 93 % or more of the plasma rT3 (Chopra, 1976; Schimmel and Utiger, 1977). The continued stepwise deiodination of T4 is also responsible for the presence of most if not all of the plasma mono- and diiodinated thyronines (Rudolph et al., 1976; Chopra et al., 1978; Sorimachi and Cahnmann, 1979). A schematic diagram of the stepwise deiodination scheme for thyroxine showing the possible steps and partially iodinated intermediates is shown in Figure 5. The individual steps in the deiodination sequence are presently thought to be catalyzed by enzymes which have a high affinity and specificity for the iodothyronine substrates.

It is proposed that the enzymes active in the conversion of T4 to T3 (5'-deiodinase) and of T4 to rT3 (5-deiodinase) are components of two distinct enzyme systems. Experiments have shown that the inner and outer ring deiodinases have different pH optimum (Cavaleri et al., 1977; Kaplan and Utiger, 1978; Sorimachi and Robbins, 1979), and that the 5'-deiodinase and the 5-deiodinase have maximal activity in the particulate and soluble fractions of cellular homogenates respectively (Cavalieri et

19

Figure 5. Schematic diagram for the stepwise deiodination of thyroxine in peripheral tissues.

The solid arrows indicate those steps catalyzed by an outer ring monodeiodinase (5'-deiodinase). The outlined arrows indicate those steps catalyzed by an inner ring monodeiodinase (5-deiodinase).

$R = -CH_2CH(NH_2)COOH$
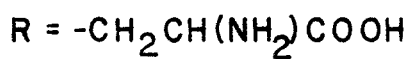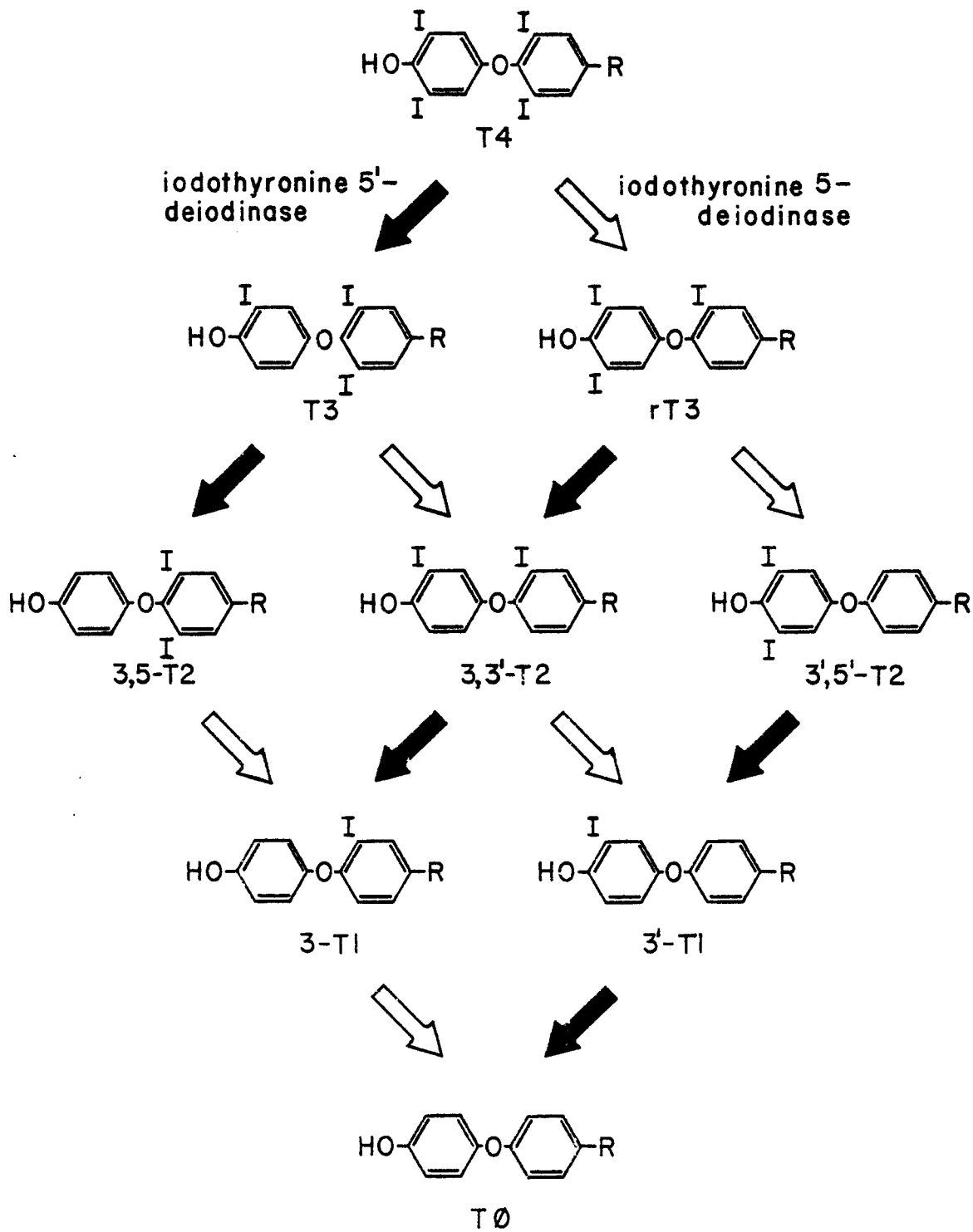
al., 1977; Chopra, 1977a). Perhaps the most convincing evidence of two distinct enzyme systems is a reciprocal relationship that exists between T3 and rT3 concentrations in normal cord umbilical serum and in serum from patients suffering from a variety of nonthyroidal illnesses (Table 1). These conditions are characterized by normal or near normal levels of serum T4, decreased levels of serum T3 and a corresponding increase in serum rT3. Were there only a single enzyme catalyzing the conversion of T4 to T3 and T4 to rT3, one would expect the serum levels of T3 and rT3 to increase or decrease in parallel.

The bidirectional conversion of T4 to T3 and rT3 offers a mechanism whereby T4 can act as a prohormone and be activated by conversion to T3 or be deactivated by conversion to rT3. Therefore, an analogy to vitamin D3 metabolism has been proposed for the metabolism of T4. For example, in the metabolism scheme for vitamin D, 25-hydroxy-cholcalciferol is converted by a double enzyme system in the kidney to either the biologically active 1,25-dihydroxy form or to the inactive 24,25-dihydroxy form of vitamin D (DeLuca et al., 1973). When $1,25-(OH)_2$ -D3 production is decreased, there is a corresponding increase in the production of $24,25-(OH)_2$-D3 and vice versa (Tanaka and DeLuca, 1974). The peripheral deiodination of T4 to T3 and rT3 is quite similar in that it is also thought to be controlled by a double enzyme system consisting of the 5-and 5'-deiodinase enzymes, and as indicated in Table 1, a decrease in the levels of T3 are associated with a corresponding increase in serum rT3 levels in a variety of "sick euthyroid" conditions.

The mechanism by which the body controls the bidirectional deiodinase system to result in decreased serum T3 levels is presently unknown.

Table 1

Reciprical Relationship of Serum T3 and rT3 Concentrations

During Non-Thyroidal Illness and in the Newborn

Study Group	TOTAL*			FREE*		
	T4	T3	rT3	T4	T3	rT3
Newborn Cord Serum	↑	⬇	⬆	↓	↓	⬆
Hepatic Cirrhosis	↓	⬇	⬆	↑	⬇	↑
Chronic Renal Failure	↓	↓	↑	↓	↓	↑
Acute Febile Illness	↑	⬇	⬆	↑	↓	⬆
Protein Calorie Malnutrition	↓	⬇	↑	—	—	—

*Increased and decreased values for total and free (dialyzable) hormone levels are based on serum concentrations for each study group as reported by Chopra and colleagues (Chopra et al., 1975a). The size of the arrows are roughly proportional to relative increases or decreases in serum hormone concentration.

It has been proposed that a decrease in the concentration of the active 5'-deiodinase, or reductions in intracellular cofactor (reduced gluta-thione, NADPH) concentrations during starvation, stress and febrile ill-ness might be responsible for the reduced iodothyronine 5'-deiodinase activity (Balsam and Ingbar, 1979; Harris et al., 1979). In addition, it has been suggested that glucocorticoids, which have an inhibitory effect on the conversion of T4 to T3, may also be involved with the reduction in 5'-deiodinase activity (Chopra et al., 1975a; Westgren et al., 1977). Increases in serum rT3, on the other hand, result from decreased degrad-ation rates as well as increased production, and inhibition of the 5'-deiodinase that metabolizes rT3 to 3,3'-T2 is thought to be a major con-tributor to increased serum rT3 levels (Kaplan and Utiger, 1978; Lumholtz et al., 1979).

Within the past 10 years, the importance of iodothyronine 5'-deiodinase in regulating serum levels of T3 and, therefore, in potenti-ating the biologic effect of thyroxine has been recognized. As a result of increased interest in the extrathyroidal metabolism of thyroxine, a major effort is currently being made by numerous groups to elucidate the biochemical properties of the 5'-deiodinase and to determine what factors affect the catalytic rate of T4 to T3 conversion.

Iodothyronine 5'-Deiodinase

The possibility that T3 might be derived from the conversion of T4 to T3 in the peripheral tissues in addition to direct thyroidal secretion was first proposed by Pitt-Rivers and colleagues in 1955 (Pitt-Rivers et al., 1955), and the first evidence of the in vitro, extrathyroidal con-

version of T4 to T3 was reported by Albright and Larsen (1959). Interest in the deiodinase system was slow to develop, however, because it had not been conclusively demonstrated in vivo that T4 was converted to T3 in peripheral tissues (Lassiter and Stanbury, 1958). Therefore, the question lay dormant until 1970, when Braverman, Ingbar and Sterling demonstrated the conversion of T4 to T3 in athyroidic patients maintained on highly purified T4 (Braverman et al., 1970). At first the peripheral conversion was proposed to be a nonenzymatic random deiodination (Surks and Oppenheimer, 1971). This proposal was widely accepted because T4 was deiodinated to T3 and rT3 in approximately equal amounts, and because in vitro assays in crude homogenate systems failed to show substrate saturation (Hüfner and Khöpfle, 1976). In 1975, Vagenakis and colleagues demonstrated that rT3 increased corresponding to a decrease in T3 in serum from fasting patients (Vagenakis et al., 1975), and interest turned once again to an enzymatic mechanism. Fisher and Sack proposed that the reaction was catalyzed by tyrosine hydroxylase (Fisher and Sack, 1975). This proposal was subsequently discounted on the basis that alpha-methyl-para-tyrosine, a specific inhibitor of tyrosine hydroxylase, did not affect the in vitro deiodination of T4 (Hüfner, et al., 1977). Finally, in 1977, Chopra demonstrated in vitro substrate saturation by T4, and demonstrated that the deiodinase activity was enzymatic in nature (Chopra, 1977a). Since 1977, the 5'-deiodinase has been studied in a variety of animal systems both in vivo and in vitro.

Iodothyronine 5'-deiodinase activity has been observed in all vertebrate species studied. In mammalian species, the enzyme is present in the highest concentrations in the kidney, liver, and pituitary tissues

(Chopra, 1977a; Cheron et al., 1980). Muscle, heart, spleen, brain, intestine, and thyroid tissues contain smaller amounts of activity (Chopra, 1977a; Ishii et al., 1981). Polymorphonuclear leukocytes (Woeber and Maddux, 1978), lymphocytes (Kvethy, 1978), and fibroblasts (Refetoff et al., 1972) also have been shown to have 5'-deiodinase activity.

Within the cell, 5'-deiodinase activity appears to be distributed throughout the cellular membranes with the microsomal and plasma membrane fractions being particularly rich in enzyme activity. After conventional subcellular fractionation (de Duve et al., 1955), the largest single fraction of T4 to T3 converting activity is the crude microsomal fraction (Visser et al., 1976b; Takaishi et al., 1979). This fraction, however, contains no more that 50 % of the total cellular activity, and results from more elaborate fractionation studies have shown that of the activity in the crude microsomal and nuclear fractions, a major portion of the 5'-deiodinase co-purifies with enzyme markers associated with the plasma membrane fraction (Maciel et al., 1979).

Iodothyronine 5'-deiodinase activity has been studied in vitro in numerous laboratories using crude membrane preparations or whole cell homogenates of various tissues. There has been no report of a successful solubilization of an active form of the enzyme, and all studies have been performed on particulate preparations of the 5'-deiodinase. The most purified particulate preparations thus far achieved have been microsomal membrane preparations which have allowed increases in specific activity of from 2-to 6-fold in comparison to the crude homogenate preparations (Visser et al., 1976b; Höffken et al., 1978; auf dem Brinke et al., 1979).

Iodothyronine 5'-deiodinase activity has been shown to be sensitive to both temperature and pH. The temperature optimum has been reported to be 37 °C (Chopra, 1977a). Preincubation of enzyme preparations for 60 minutes at 24 °C, 37 °C, and 56 °C resulted in the loss of 15 %, 43 %, and 98 %, respectively of the recoverable activity (Chiraseveenprapund et al., 1978). Optimum pH values at 37 °C have been reported in the range of 6.0 to 7.35 (Höffken et al., 1977; Hüfner et al., 1977; Chopra, 1977a).

Iodothyronine 5'-deiodinase activity has been reported to be linear with time (37 °C) during the initial 45 to 60 minutes of incubation (Chiraseveenuprapund et al., 1978; Sorimachi and Robbins, 1979). Linearity with protein concentration, however, has not been observed for the crude enzyme preparations (Leonard and Rosenberg, 1977; Sorimachi and Robbins, 1979).

The current literature has been inconsistent concerning the effects of various ions on the activity of the 5'-deiodinase, however, it has been generally agreed that there are no requirements for metal cofactors (Kaplan and Utiger, 1978) and that the ions: Ca^{2+}, Mg^{2+}, Li^+, Na^+, and K^+ do not have a major inhibitory or stimulatory effect (Visser et al., 1976b; Chopra, 1976; Sorimachi and Robbins, 1979). Heavy metal ions including: zinc, mercury, lead, and silver, on the other hand, have been shown to be potent dose-dependent inhibitors of the 5'-deiodinase (Chopra, 1977b).

It has recenlty been observed that several sulfhydryl agents including dithiothreitol (DTT), mercaptoethanol, cysteine, and reduced glutathione are potent stimulators of iodothyronine 5'-deiodinase activity

(Chopra, 1977b; Harris et al., 1979). On the other hand, oxidized gluta-
thione has been shown to inhibit monodeiodination (Chopra et al., 1978).
In addition, the sulfhydryl oxidizing agent, diamide, and the sulfhydryl-
binding agent N-ethylmaleimide have also been shown to be potent inhibi-
tors of the 5'-deiodinase (Chopra et al., 1978). Based on these data and
the demonstration of thiol dependent activity in microsomal preparations,
it has been proposed that the presence of thiol-containing compounds is
essential for 5'-deiodinase activity (Chopra et al., 1978; Sorimachi and
Robbins, 1979; Leonard and Rosenberg, 1980b). The naturally occuring,
endogenous cofactor has been proposed to be reduced glutathione (Visser
et al., 1976b; Chopra et al., 1978; Harris et al., 1979). NADPH has been
proposed to have an activating effect in vivo as a reductant for regener-
ating reduced glutathione (Balsam and Ingbar, 1978; Chopra et al., 1978).
No other chemical activators or dialyzable cofactors of the 5'-deiodinase
have been reported.

The stimulation of T4 to T3 conversion by the presence of thiol
group-containing compounds has been proposed to result in part from their
efficiency in maintaining the 5'-deiodinase in its active conformation
(Chopra et al., 1978). It is suggested, therefore, that the enzyme may
exist in an inactive conformation characterized by disulfide linkages
between cysteine residues, and that in the active conformation these
disulfide bonds are reduced. In addition, it has been proposed that the
thiol group-containing compounds may act as cofactors and participate
directly in the reductive deiodination of T4 (Chopra et al., 1978; Vis-
ser, 1979).

In addition to sulfhydryl-binding and sulfhydryl-oxidizing agents,

the 5'-deiodinase is inhibited in a dose-dependent manner by various T4 metabolites including: rT3, 3',5'-T2, and 3,3'-T2 as well as by propyl-thiouracil, sodium salicylate, and some iodinated x-ray contrast agents (Visser et al., 1978; Chopra et al., 1978; Chopra et al., 1980). Pro-pranolol, a beta-adrenergic blocking agent, has also been reported to have an inhibitory effect on the in vitro conversion of T4 to T3 (Lum-holtz et al., 1979; van Noordan et al., 1979). Reverse T3, the most potent inhibitor of the various agents studied so far, has been reported to be both a competitive inhibitor with K_I's of 0.045 μmol/L (Chopra, 1977a) and 0.0045 μmol/L (Kaplan and Utiger, 1978), and to be a non-competitive inhibitor (K_I = 0.02 μmol/L (Hüfner, 1977). Propylthiouracil has been reported to be an uncompetitive inhibitor with respect to T4 concentration, a competitive inhibitor with respect to dithiothreitol concentration (Visser et al., 1978), and an allosteric effector (Kaplan and Utiger, 1978). Sodium salicylate and the iodinated x-ray contrast agents have been reported to be competitive inhibitors (Chopra et al., 1978; Chopra et al., 1980). The conversion of T4 to T3 is reported not to be affected by dexamethasone (Kaplan and Utiger, 1978); by T3 (Hüfner et al., 1977); by 3,5-T2, iodotyrosines; nor by methylated, brominated, or chlorinated analogs of rT3 (Chopra et al., 1978).

Iodothyronine 5'-deiodinases catalyze the conversion of rT3 to 3,3'-T2 as well as the conversion of T4 to T3 (See Figure 5). The simi-larities in these 2 steps has led to an ongoing debate over whether the same enzyme catalyzes both reactions. Several findings suggest that both steps are carried out by the same enzyme. First, the tissue distribution and subcellular location of both 5'-deiodinases is similar (Chopra et

al., 1978). Secondly, both activities require the presence of thiol-group-containing compounds for activity and both are inhibited by propyl-thiouracil in an uncompetitive manner (Chopra et al., 1978). Thirdly, rT3 is an inhibitor of T4 to T3 conversion and T4 is an inhibitor of the rT3 to 3,3'-T2 deiodinase (Visser et al., 1979b). In addition, both activities have been shown to decrease during fasting and hypothyroidism, and to increase in response to hyperthyroidism (Kaplan and Utiger, 1978). There are, however, several findings that do not support a one enzyme theory. For example, the Km of rT3 for the 5'-deiodinase (65 nmol/L rT3) is approximately 40-fold lower than the Km of T4 (3.0 μmol/L T4), and the optimum pH for the T4 to T3 and rT3 to 3,3'-T2 activities are 6.5 and 8.0, respectively (Visser et al., 1979b). Moreover, while the character-istically low serum T3 and T4 to T3 converting activity in tissues of the fetus increase to normal adult levels within a few hours of birth, the characteristically high serum rT3 levels in the fetus do not decrease to adult levels until the second week of postnatal life (Maciel et al., 1979). If the outer ring deiodinases for T4 and rT3 were identical, one would expect the changes to be more nearly synchronized. Thus, while the two activities are similar in some respects, the question of similarity versus identity awaits purification of the 5'-deiodinase.

Kinetic studies utilizing crude membrane preparations have been performed by several groups to determine the Michaelis-Menten constant (Km) and the velocity for the conversion of T4 to T3. The apparent Km for the 5'-deiodinase of these crude preparations has been reported to be in a range of from 0.8 to 7.7 μmol/L T4 (Hüfner et al., 1977; Chopra, 1977a; Kaplan and Utiger, 1978; auf dem Brinke et al., 1979), and the

velocities of the most purified membrane preparations have been reported typically to be in a range of from 0.5 to 1.2 pmol T3 $min^{-1}mg^{-1}$ (Takaishi et al., 1979; Leonard and Rosenberg, 1980b).

Iodothyronine 5'-deiodinase is thought to catalyze the reductive deiodination of T4 (Visser et al., 1976b). It is proposed that a

$$T4 + 2 \ R\text{-}SH \longrightarrow T3 + HI + R\text{-}SS\text{-}R$$

thiol-group-containing compound (R-SH) acts as a second substrate and that the products of the reaction are T3, HI and the oxidized thiol. It is also proposed that the conversion of rT3 to 3,3'-T2 occurs by the same mechanism (Visser et al., 1979b).

When the kinetics of the outer ring deiodination of rT3 to 3,3'-T2 were examined using different concentrations of DTT over a range of rT3 concentrations, the concentration of DTT added to the enzyme assay mixture was found to affect both the Km and maximum velocity such that a family of parallel lines was generated when a double reciprical plot of velocity versus rT3 concentration was plotted at differing concentrations of dithiothreitol (Visser, 1979). This type of kinetic data is characteristic of a two-step or ping-pong mechanism in which the enzyme oscillates between two stable forms (Cleland, 1963). Visser and colleagues suggested that the conversion of rT3 to 3,3'-T2 might occur by a ping-pong mechanism where (E-SH) represents the active form of the
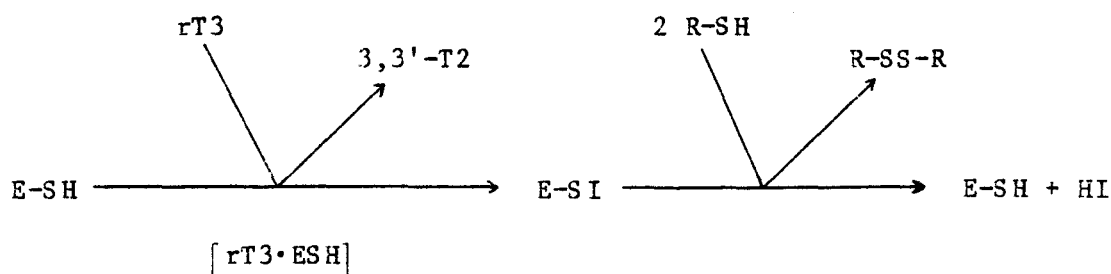
enzyme and (E-S I) represents a stable intermediate sulphenyl iodide (RS-I) form of the enzyme (Visser et al., 1979b). According to this type of mechanism, the deiodination reaction occurs in two steps. The first step was proposed to involve a concerted mechanism whereby a primary attack of the rT3 substrate by an enzyme sulfhydryl group resulted in a enzyme-hormone complex (rT3·E-SH) which subsequently broke apart releasing free 3,3'-T2 from the iodinated enzyme (E-S I) (Visser et al., 1979b). In the second step, the oxidation of two thiol equivalents reduced the enzyme back to its original active form and allowed the release of free hydrogen iodide (HI).

The proposed ping-pong mechanism has been supported by the demonstration that propylthiouracil (PTU) inhibits the 5'-deiodinase uncompetitively with respect to rT3 and competitively with respect to dithiothreitol (Visser, 1979). It has been demonstrated that the reaction of iodine with protein yields sulphenyl iodides which are highly reactive towards thiouracil derivatives (Cunningham, 1964). Competition between PTU and dithiothreitol for the sulphenyl could, therefore, explain the competitive inhibition with respect to dithiothreitol. By binding to the sulphenyl, PTU would effectively lower the concentration of active enzyme available for the deiodination of rT3, and this could explain the apparent uncompetitive inhibition with respect to rT3. These results are consistent with the formation of a sulphenyl iodide enzyme intermediate.

The T4 5'-deiodinase is also inhibited in a competitive manner by PTU with respect to T4 (Chopra, 1977a). Therefore, it has been proposed that the deiodination of T4 to T3 also occurs by a ping-pong mechanism (Visser et al., 1979b). Other reports, however, have suggested that the

Km of the T4 5'-deiodinase is not affected by the concentration of dithi-
othreitol (Chopra, 1977b). If this is true, it would indicate that the
conversion of T4 to T3 does not occur by the ping-pong mechanism.

Due to the particulate nature of the iodothyronine 5'-deiodinase,
purification of the enzyme requires membrane extraction. Preliminary
studies have shown that solubilization of the 5'-deiodinase from rat
liver microsomal preparations requires detergent concentrations suffi-
cient to result in extensive membrane dissassembly (Takaishi et al.,
1979). Therefore, instead of being adsorbed to the membrane surface (ex-
trinsic protein) or being included within luminal cavities of microsomal
vesicles (matrix protein), the iodothyronine 5'-deiodinase was found to
be an integral component (instrinsic protein) of rat liver microsomal
membranes.

Previous attempts to solubilize the 5'-deiodinase from rat liver
and kidney membranes (Takaishi et al., 1979; Leonard and Rosenberg,
1980a, 1980b) and from beef liver membranes (Tseng and Ltham, 1981) de-
monstrated that deoxycholate was effective in solubilizing approximately
60 % of the deiodinase from cellular membranes, however, the enzyme was
inactive in the presence of even small amounts of the detergent (Leonard
and Rosenberg, 1980) and removal of deoxycholate by QAE-Sephadex chroma-
tography, dialysis or adsorption techniques was required to demonstrate
activity in solubilized fractions. After detergent removal, the 5'-
deiodinase could be stored for 3 days at 4 °C, or for 1 month at -70 °C
with little or no loss in activity (Leonard and Rosenberg, 1980b). The
extracted enzyme was poorly soluble in aqueous buffers, however, and the
removal of detergent resulted in the formation of macromolecular aggre-

gates of the enzyme. These aggregates were excluded from Sepharose 6B-CL
when gel filtration was performed in the absence of detergent, and they
were shown, by isopycnic banding to have a membrane-like density of ap-
proximately 1.17 g/mL (Leonard and Rosenberg, 1980a).

The membrane-like density is suggestive of the presence of phospho-
lipid and, in fact, the presence of phospholipid has been demonstrated to
be essential for catalytic activity. Phospholipase A_2 treatment of the
solubilized enzyme resulted in a concentration and time dependent loss of
T4 to T3 converting activity, and the addition of soybean phospholipid
prevented the lipase inactivation in a competitive manner (Leonard and
Rosenberg, 1980a).

Gel filtration of the solubilized iodothyronine 5'-deiodinase in
the presence of deoxycholate allowed elution of T4 to T3 converting acti-
vity after the void volume. The molecular weight of the deoxycholate
solubilized 5'-deiodinase from rat kidney membranes was estimated by
Sepharose 6B-CL chromatography in the presence of Na deoxycholate to be
approximately 85,000 (Leonard and Rosenberg, 1980a). A 5'-deiodinase
enzyme complex solubilized from beef liver membranes has been reported to
have 2 included components when chromatography was performed on agarose
0.5 M in the presence of Na deoxycholate. The apparent molecular weights
of the 2 components were estimated to be 95,000 and 200,000 (Tseng and
Latham, 1981).

Attempts to purify the solubilized iodothyronine 5'-deiodinase in
the presence of deoxycholate have resulted in extremely low yields of ac-
tive enzyme. Gel filtration chromatography, for example, resulted in
yields of less than 10 % (Leonard and Rosenberg, 1980b). The poor re-

covery of activity was found to be due in part to delipidation during gel filtration as evidenced by a 5-fold increase in percent yield following the addition of membrane lipid extracts (Leonard and Rosenberg, 1980b). In addition to attempts to purify the 5'-deiodinase by gel filtration chromatography, the mention of a preliminary attempt to purify the 5'-deiodinase by adsorption chromatography on QAE-Sephadex and by fractionation with ammonium sulfate has been reported in abstract form (Tseng and Latham, 1981). To date, no other purification attempts have been published and no successful purification of the 5'-deiodinase has been reported.

The Present Study

A large body of evidence in the literature supports the existence of a specific membrane-bound iodothyronine 5'-deiodinase that catalyzes the conversion of T4 to T3. There is also substantial evidence to suggest that T3 is several times more biologically active than T4. Therefore, the 5'-deiodinase has recently been the subject of intense investigation due to its potential role in the pathogenesis and treatment of thyroid disease. The present study was undertaken in the interest of illuminating the physical and enzymatic characteristics of the iodothyronine 5'-deiodinase.

At the present time, very little information is available regarding the physical properties of the 5'-deiodinase, and there is considerable disagreement among the various investigations of the enzyme's catalytic activity. This is due, in part, to the fact that the enzyme has only been studied in crude preparations of whole cell homogenates and micro-

somal membranes. I now report a technique for the membrane extraction of iodothyronine 5'-deiodinase, and the development of a multistep purification procedure. The purification procedure was used to prepare purified thyroxine 5'-deiodinase from rat liver membranes for use in the investigation of physical properties and kinetic characteristics.

Physical characterization studies included the estimation of apparent molecular weight and the determination of isoelectric point for the rat liver iodothyronine 5'-deiodinase.

Catalytic characterization studies involved the determination of optimum conditions for enzymatic activity, the determination of Km and V_{Max}, and a study of inhibition by several known inhibitors including reverse T3, propylthiouracil, iodipamide, sodium salicylate, and propranolol.

A ping-pong mechanism has been proposed for the 5'-deiodination of rT3 to 3,3'-T2 (Visser et al., 1979b). In the present study, kinetic assays utilizing the purified rat liver 5'-deiodinase were performed for the conversion of T4 to T3 to determine if the deiodination of T4 might also occur by a ping-pong type mechanism.

Similarities in the subcellular location and catalytic effectors of the 5'-deiodinase that converts rT3 to 3,3'-T2 and the 5'-deiodinase that converts T4 to T3 have led to the hypothesis that the same enzyme catalyzes both reactions (Chopra et al., 1978; Visser et al., 1979b). There are, however, several findings that do not support a one enzyme theory such as differences in pH optimum, substrate binding constants and the in vivo metabolism of T4 and rT3 in the neonate (Maciel et al., 1979). In the present study, kinetic assays were performed with the purified

iodothyronine 5'-deiodinase to determine if the T4 to T3 converting enzyme also carried out the monodeiodination of rT3 to 3,3'-T2.

CHAPTER II

MATERIALS AND METHODS

Materials

Rat liver tissue was obtained from male and female, Sprague-Dawley laboratory rats maintained on Purina rat chow and tap water ad libitum. Groups of from 2 to 10 rats weighing 100-250 g each were anesthetized with chloroform and exsanguinated by cutting the portal vein and hepatic artery. The liver was removed, sliced into small fragments, rinsed in cold saline (0 °C) and placed into 10 mL vials. Liver tissue prepared in this manner could be stored 6 months or longer at -60 °C with no effect on the specific activity of the iodothyronine 5'-deiodinase. The non-radioactive substrate L-thyroxine (T4), was obtained from Sigma Chemical Co., St. Louis, MO. as the highly purified free acid, and was used without further purification. Other substrates, ^{125}I-labeled T4 purchased from Nuclin Diagnostics, Northbrook, IL., and ^{125}I-labeled reverse T3 (rT3) from Amersham-Searle, Arlington Heights, IL. were purified by Sephadex G-25 chromatography (Green, 1972) prior to use. The reduced thiol-group-containing cofactors: L-cysteine, glutathione, 2-mercapto-ethanol, DL-dithiothreitol and DL-6,8-thioctic acid amide (lipoamide) were obtained from Sigma. The inhibitors and related compounds: methimazole, demamethasone, DL-propranolol, sodium diatrizoate (Hypaque) and 6-n-propyl-2-thiouracil (PTU) were purchased from Sigma. Sodium salicylate was obtained from Matheson, Coleman and Bell, Norwood, OH., and iodipamide (Cholografin) was purchased from Squibb and Sons, Princeton, NJ.

Diethylamineothyl (DEAE) Cellulose (medium mesh), Concanavalin A (Con A) Sepharose 4B, Sepharose 4B, 6B-CL, Sephacryl S-300 superfine (sf), Sephadex G-25 medium and Sephadex G-25 superfine were obtained from Sigma. Carboxymethyl (CM) cellulose (CM 22) was a product of Whatman, Clifton, NJ. The sulfhydryl affinity resin, Affi-gel 401 and the hydroxylapatite resin were purchased from Bio·Rad, Richmond, CA. The organomercurial Sepharose 4B resin was synthesized according to the procedure of Sluyterman and Wijdeness (1974).

Buffers used throughout the purification of the rat liver iodothyronine 5'-deiodinase included: homogenate buffer (A) 5 mmol Tris-HCL, 250 mmol sucrose, pH 8.0; homogenate buffer (B) 25 mmol KPO_4, 50 mmol NaCl, 1 mmol EDTA, 0.02 % NaN_3, 10 mmol 2-mercaptoethanol (BME), pH 6.5; extraction buffer (C) 2 mmol KPO_4, 10 mmol NaCl, 1 mmol EDTA, 0.02% NaN_3, 10 mmol BME, pH 7.5; column buffer (D) 2 mmol KPO_4, 10 mmol NaCl, 1 mmol EDTA, 0.02 % NaN_3, 10 mmol BME, 0.1 % Nonidet P-40, pH 7.6; elution buffer (E) same as buffer D except for the content of NaCl which was increased to 500 mmol NaCl; column buffer (F) Same as buffer D except pH 6.5; elution buffer (G) 500 mmol KPO_4, 150 mmol NaCl, 1 mmol EDTA, 0.02 % NaN_3, 10 mmol BME, 0.1 % Nonidet P-40, pH 6.5; gel filtration column buffer (H) 25 mmol KPO_4, 50 mmol NaCl, 1 mmol EDTA, 0.02 % NaN_3, 10 mmol BME, 0.1 % Nonidet P-40, pH 6.5.

The detergents, Triton X-100 and deoxycholate, and pancreatic lipase were purchased from Sigma. Nonidet P-40 was obtained from Bethesda Research Laboratories, Rockville, Md. A test kit containing the zwitterionic detergents, derivatives of N-hexadecyl-N,N-dimethyl-3-amino-1-propane sulfonates, were obtained from Calbiochem Behring Corp., La

Jolla, CA.

All reagents used for the quantitation of T3 by radioimmunoassy were of the highest purity commercially available. Rabbit antisera to T3 was obtained from Endocrine Science Laboratories, Tarzana, CA. Calbiochem-Behring was the source of goat antibody to rabbit gamma globulin (GARGG). Non-radioactive 3,3',5-triiodo-L-thyronine (T3) was supplied by Sigma. ^{125}I-labeled T3 with a specific activity of approximately 2400 μCi/μg was purchased from Nuclin Diagnostics. Reagent alcohol (95% ethanol) was the product of Mallinkrodt, St. Louis, MO. Human serum pools were kindly provided by the clinical laboratory of Loyola University Medical Center, Maywood, IL. Activated charcoal "Norit A" was purchased from Matheson, Coleman and·Bell.

Other reagents: bovine serum albumin (Cohn fraction V powder), Coomassie Brilliant Blue G-250, and 3,5'-diiodo-L-thyronine (3,5-T2) were purchased from Sigma. Carrier ampholytes (3/10) were the product of LKB Instruments, Inc., Rockville, MD. Purified proteins used for the calibration of gel filtration columns were obtained from Pharmacia, Piscataway, NJ.

The following specialized equipment was utilized: A Potter-Elvehjem tissue homogenizer (55 mL flask, Kontes Glass Co., Vineland, NJ.) with the stan-dard teflon pestle (0.007 inch clearance) and a pestle that was turned on a lathe to a clearance of 0.024 inches (Fleischer and Kervina, 1974). Dounce homogenizer (40 mL flask; Kontes Glass Co.) with standard large clearance (A) and small clearance (B) pestles; miracloth filtration material (Calbiochem); Sorvall Superspeed (RC2-B) refrigerated centrifuge with the SS-34 rotor (r_{min} = 5.72 cm, r_{max} = 10.80 cm);

Beckman preparatory L5-65 ultracentrifuge with the SW-27 head (r_{min} =

7.53 cm, r_{max} = 16.12cm); Beckman "Microfuge" (Model 152) with 400 μL

disposable microtubes (Walter Sarstedt, Inc., Princeton, NJ); Amicon

Corp., Chicago, IL) washed according to the procedure of Brewer (1974),

and stored prior to use at 4 °C in deionized water with 0.02 % NaN_3

as a bacteriostatic agent; LKB electrofocusing column (110 mL Ampholine

column, #8100); LKB gradient mixer (Model 8121); D.C. power supply

(Buchler Instruments, Fort Lee, NJ); and a TI-59 programmable calculator

with Master Library Module software program (Texas Instruments, Inc.,

Dallas, TX).

Methods

Preparation of Rat Liver Homogenates

Homogenates of the rat liver tissue were prepared with a motor

driven Potter-Elvehjem homogenizer. The frozen tissue was thawed in 4

volumes of homogenate buffer A or homogenate buffer B and all subsequent

steps were carried out at 0-4 °C. The initial tissue disruption was per-

formed with 3 passes of pestle A and cellular homogenization was com-

pleted with 3 to 5 passes of pestle B. The resulting 20 % homogenate was

filtered through a single layer of miracloth to remove unbroken cells and

tissue fragments. The filtrate (Fraction A), containing approximately 28

mg protein/mL was stored in 10 mL aliquots at -60 °C or used directly as

the source of the iodothyronine 5'-deiodinase for purification studies

and for subcellular fractionation studies.

In Vitro Studies of Thyroxine 5'-Deiodinase Activity

T4 to T3 converting activity was measured by a modification of methods described previously (Chopra, 1977a; Maciel et al., 1979; auf dem Brinke et al., 1979). In summary, aliquots of samples suspected of containing thyroxine 5'-deiodinase activity were incubated with non-radioactive T4 for the time periods given in RESULTS, and the amount of T3 generated was measured by radioimmunoassay (RIA) performed on a ethanol extract of the incubation mixture. Under standard reaction conditions, the following reagents were added in 12 x 75 mm polystyrene culture tubes in the order given: 1) 320 μL of 150 mmol/L phosphate buffer containing 1 mmol/L EDTA, pH 6.5; 2) 50 μL of 200 mmol/L dithiothreitol, made fresh in the same buffer; 3) 100 μL of enzyme preparation; 4) 30 μL of substrate solution containing 250 μg T4/mL deionized water. The final 500 μL volume containing 20 mmol/L dithiothreitol, 19.3 μmol/L T4, and 0.01-0.5 mg protein buffered to pH 6.5 was mixed gently by swirling and placed in a water bath thermostated to 37 °C. The reaction was stopped following a 30 min incubation (standard reaction conditions) by extraction of the incubation mixture with 2 volumes (1.0 mL) of 95 % ethanol. This concentration of ethanol destroyed enzymatic activity completely and afforded a 96-100 % recovery of iodothyronines. The assay tubes were then tightly capped, mixed by inversion to facilitate complete extraction of the T3, and the precipitated protein was pelleted by centrifugation for 10 min (1,000 x g). Each assay contained zero incubation tubes (blanks) which were handled exactly as described above except that the 100 μL aliquot of sample was added following incubation and immediately prior to the extraction step. The T3 content of the blank tubes was considered to represent T3 formed from the spontaneous deiodination of T4, T3 contained

in the commercial T4 preparation, and "apparent" T3 resulting from the cross-reactivity of T4 in the T3 radioimmunoassay. The blank varied from 4.5 to 7.2 ng T3/tube when 7.5 µg T4 was used in the assay. This value was subtracted from T3 measured in the test samples to determine the amount of T3 produced from the enzymatic deiodination of T4. The results were expressed in units of activity which were defined as the quantity of enzyme that transformed 1 pmol T3 per min at 37 °C.

To study the effect of various conditions on 5'-deiodinase activity, changes in standard assay procedure were made as indicated in the text and Figure legends. The addition of various agents to the enzyme assay incubation mixture were made by replacing aliquots of enzyme assay buffer with equal volumes of solutions containing the test agents. When the effects of various salts were studied, the phosphate buffer was made up without EDTA. When studying the effect of various cofactors, solutions of the various sulfhydryl agents were used in place of the dithiothreitol solution.

Kinetic studies on crude and purified preparations of the 5'-deiodinase were performed using standard assay conditions with varying substrate or cofactor concentrations. Data were generated under conditions of initial reaction rates and plotted by the method of Lineweaver and Burk (1934). For the determination of apparent Km and Vmax were determined from replots of the primary data (Dixon and Webb, 1979a). K_I's for the various inhibitors were determined from a replot of apparent Km or Vmax values determined in the presence of fixed inhibitor concentrations (Lineweaver and Burk, 1934; Dixon and Webb, 1979d).

Enzyme assays performed on samples from the subcellular fraction-

ation study were carried out in the absence of detergent. Unless indicacated otherwise, all other assays were done on samples containing 0.1 % Nonidet P-40. Sample volumes of 100 μL were diluted to a final 500 μL in the reaction mixture resulting in a final 0.02 % detergent concentration. This concentration was slightly above the critical micellular concentration (CMC) of 0.017 % for Nonidet P-40 (Helenius and Simons, 1975). For samples not containing 0.1% Nonidet P-40, detergent was added or samples were diluted in homogenate buffer B.

Due to the nonlinearity of activity with high protein concentration, serial dilutions of the crude fractions (A through E) were assayed for 5'-deiodinase activity in order to relate their activity to that of the more purified fractions. Maximum protein concentrations used were less than 5 mg/mL. Fraction III and other fractions of high specific activity were diluted in buffer H to protein concentrations no lower than 0.10 mg/mL. All enzyme specific activities were based on protein determinations by Coomassie Blue dye-binding (Bradford, 1976) using bovine serum albumin as the standard.

T3 Radioimmunoassay

3,3',5'-Triiodo-$\underline{L}$-thyronine (T3) quantitation was performed by a double antibody radioimmunoassay technique very similar to that described previously for the quantitation of T4 (Collins et al., 1980). Reaction mixtures were pipetted into 400 μL disposable microtubes in the following order: 1) 120 μL of sample (20 μL of enzyme extract + 100 μL charcoal stripped serum); 2) 100 μL T3 Trace (^{125}I-T3 sufficient to generate approximately 24,000 cpm/0.1 mL prepared in 100 mmol/L barbital buffer,

pH 8.6 containing 0.5% bovine serum albumin, 0.5% thimerosal and 0.6 mL of rabbit serum per 100 mL); 3) 100 µL T3 Antibody (lyophilized rabbit T3 antiserum reconstituted in the same buffer as used for dilution of T3 Trace with the exception of deletion of rabbit serum; optimal dilution should allow binding of 45-55 % of the ^{125}I-T3 in the absence of non-radioactive T3); 4) 25 µL Precipitating Antibody (goat anti-rabbit gamma-globulin (GARGG) antibody reconstituted according to manufacturers recommendation). Following the addition of GARGG, the contents of the assay tubes were mixed and allowed to incubate for 16 h at room temperature. The tubes were then centrifuged for 2 min (10,000 x $\underline{g}$) in a Beckman microcentrifuge to precipitate the bound hormone complex, and the unbound hormone was removed by aspiration. The cpm of ^{125}I-T3 remaining in the precipitant were determined by counting each tube in an automatic gamma counter for a period of 60 sec. T3 Standards (0,25,50,75,100,150,200,-300,500 and 600 ngT3/100 mL) were made up in charcoal stripped serum and were run with each assay by replacing the 100 µL of charcoal stripped serum in step (1) with 100 µL of the respective T3 Standard. In a like-wise manner, control pools were assayed to determine inter-and intra-assay variation. A total counts tube, consisting of 100 µL T3 Trace, and a nonspecific binding (NSB) tube, set up as described above using bar-bital buffer in the place of T3 Antibody for the zero T3 standard, were also added to each assay. Data for the standard curve were linearized in the form of a logit-log plot (Rodbard et al., 1968) with the use of a TI-59 programmable calculator. The NSB (<5 %) and the precision (inter-assay and intra-assay coefficients of variation <8 %) compared favorably to that reported previously (Collins et al., 1980).

Extracts of the enzyme assay containing 63.3 % ethanol were added to the RIA incubation mixture in 20 μL aliquots. This volume resulted in a final 3.7 % concentration of ethanol and resulted in an increase in the slope of the standard curve of from -2.6 to -2.4. In order to minimize the effects of ethanol on measurements of T3 by the RIA procedure, 20 μL aliquots of the incubation mixture were consistently used through-out this study, and 20 μL aliquots of 63 % reagent alcohol were added to standard and control tubes as described. Since the ethanol extract contained little or no protein, as evidenced by a lack of precipitant in the presence of 10 % trichloroacetic acid, use of 63 % reagent alcohol alone to treat the standards and controls was considered sufficient to render the unknowns comparable to the standards (Chopra, 1977a). The presence of Nonidet P-40 in the ethanol extract (0.0067 %) resulted in a final detergent concentration of 0.0004 % in the RIA mixture, and was found not to have a significant effect on the performance of the radioimmunoassay. Any effects of other non-T3 substances on the RIA were taken into account by measuring T3 in the zero-incubation (blank) tubes.

The sensitivity of the assay varied from 15 to 25 pg T3 per tube, as determined by the amount of T3 required to reduce the number of cpm of the zero standard by 10 %. This allowed detection of as little as 1.35 ng T3 in the enzyme assay incubation mixture. This amount was a very small fraction (0.02 %) of the 7.5 μg T4 added per enzyme assay.

Subcellular Fractionation

Subcellular fractions of the rat liver homogenate (Fraction A) were prepared by the method of Aronson and Touster (1974), with slight modifi-

cation. Fraction A, suspended in homogenate buffer A, was centrifuged at 1,000 x g for 10 min. The supernatant was removed by pipette and the pellet was rehomogenized in buffer A. This step was repeated twice and the three supernatants were combined and designated as the cytoplasmic extract (Fraction E). The washed pellet, resuspended in 57 % sucrose, was labeled as the nuclear fraction. In order to sediment the nuclei, the nuclear fraction was centrifuged at 33,000 x g for 7.5 min. Following centrifugation, the supernatant fluid was removed by aspiration and the fluffy, dark brown material layered over a small firm pellet of tan material was decanted. This dark brown material was suspended in 3 volumes of 57 % sucrose and labeled as Fraction N (nuclei). The supernatant and the tan pellet were discarded. The cytoplasmic extract (Fraction E) was also centrifuged at 33,000 x g for 7.5 min to obtain the mitochondrial and lysosomal pellet (Fraction M + L). The remaining 33,000 x g supernate was centrifuged at 78,000 x g for 100 min. The resulting pellet, resuspended in 4 volumes of 57 % sucrose, was labeled as Fraction P (microsomal fraction). The 78,000 x g supernatant was labeled as Fraction H (cell cytosol).

Plasma membrane fractions were obtained from the nuclei (Fraction N) and microsomal (Fraction P) preparations by high speed centrifugation in a discontinous sucrose gradient according to the procedure of Avonson and Touster, 1974. In short, 15-18 mL of the suspended crude nuclei (N) or 13-15 mL of the crude microsomes (P) were placed in the bottom of cellulose nitrate centrifuge tubes for the Beckman SW-27 rotor. Fifteen milliliters of 37.2 % sucrose were then carefully layered over the nuclei sample without mixing of the layers, and a third layer of 250 mmol/L

sucrose was added in the same manner for a total volume of 38.5 mL. On the microsomal fraction were layered 17 mL of 34 % sucrose and a layer of 250 mmol/L sucrose to a final volume of 38.5 mL. The discontinuous gradients were then centrifuged at 75,500 x g (24,000 rpm) for 16 h. The electronic brake was used to decelerate the rotor at the end of the run. Fractions from each run were collected. Four fractions were obtained from the nuclei: The first, a layer of congealed fat (N_1), floated at the top of the gradient. A plasma membrane band (N_2) consisting of small pieces of a tan material appeared at the interface of the 250 mmol/L and 37.2 % sucrose layers. The 37.2 % layer was removed as the third fraction. This band (N_3) was reported to consist of mitochondria and segments of plasma membrane. The bottom portion containing the nuclei was the final fraction (N_4). Four fractions were also obtained from the microsomal material: The first, a layer of congealed fat (P_1) floated at the top of the gradient. The plasma membrane fraction (P_2), a thick band of white material, formed at the interface between the 250 mmol/L and 34 % sucrose layers. The 34 % sucrose layer (P_3) appeared faintly cloudy and contained a mixture of plasma membranes and microsomes. The microsomal membrane fraction (P_4) appeared at the top of the 57 % sucrose sample layer.

All resuspensions, following centrifugation steps, were done with the aid of a Dounce type tissue homogenizer. Homogenization was accomplished with from 2 to 4 complete strokes of the "A" pestle through the homogenate suspensions. All steps were carried out at 0-4 °C. Buffer flasks were pre-chilled on crushed ice. The homogenate solutions were kept on ice at all times except during centrifugation steps which were

carried out in refrigerated centrifuges. All sucrose solutions were made in 5 mmol/L Tris buffer and titrated to pH 8.0 (23 °C) with 1 mol/L HCL prior to their final dilution. Buffers were checked for sucrose concentrations by refractometry. The percent sucrose, as read directly from a Seitz refractometer at 25 °C, was determined to be ± 0.05 % of the concentrations given. The complete separation procedure required approximately 48 hours from start to finish and aliquots saved from each fraction were dialyzed against a 10-fold dilution of buffer B prior to protein quantitation and enzyme activity studies. All enzyme assays were run concurrently to determine the relative specific activity of thyroxine 5'-deiodinase in each fraction.

Preparation of Crude Rat Liver Membranes

Fraction A homogenate prepared in buffer B and stored frozen in 10 ml aliquots for up to 6 months at -60 °C was thawed rapidly in a 37 °C water bath with continual agitation. Just prior to complete thawing, the vials were placed in ice water. The thawed homogenate was then centrifuged at 37,000 x $\underline{g}$ for 1 h in a Sorvall SS-34 rotor. The cytosol (Fraction B) was decanted and saved for further analysis. The firm light brown membrane pellet and a clear yellow gel at the bottom of the centrifuge tube were resuspended in homogenate buffer B to initial volume and centrifuged a second time at 37,000 x $\underline{g}$ for 1 h. Following the second centrifugation, the clear supernate was discarded and the crude membrane pellet was resuspended in initial volume in extraction buffer C and labeled as Fraction C.

Solubilization of Iodothyronine 5'-Deiodinase

Several extraction techniques were attempted in preliminary studies to achieve a selective solubilization of the rat liver iodothyronine 5'-deiodinase from Fraction C. These techniques were performed according to commonly used membrane extraction procedures (Morton, 1955; Penefsky and Tzagoloff, 1971) and included: acid and alkali extraction, freezing and thawing, extraction with organic solvents (n-butanol and ethanol), enzymatic digestion with pancreatic lipase, and detergent solubilization with deoxycholate, zwittergents (sulfobetaines), Triton X-100, and Nonidet P-40. A description of the conditions utilized is given in the text and figure legends of the Results section. The method chosen in the present study for solubilization of the membrane bound 5'-deiodinase was solubilization with Nonidet P-40. All steps were carried out at 0-4 °C.

Commercially prepared Nonidet P-40 (NP-40) was used without further purification and a 10 % working solution was prepared by diluting the commercial reagent with deionized water. This solution could be kept for several weeks at room temperature without the formation of a precipitate.

Solubilization of T4 5'-deiodinase activity was carried out in two steps. In the first step, NP-40 was added to Fraction C in a dropwise manner with stirring to a final concentration of 0.05 %. The detergent-membrane mixture was stirred for an additional 10 min, and homogenized in the Dounce homogenizer with 10 complete strokes of pestle A, and 2 strokes with pestle B. The suspension was then centrifuged at 37,000 x g for 1 hr in the Sorvall centrifuge with the SS-34 rotor. The 0.05 % NP-40 extract (Fraction C_2) was removed by pipette and discarded, and the

remaining membrane (Fraction C_1) was resuspended in 4 volumes of buffer

C. In the second step, NP-40 was added to Fraction C_1 to a final concen-

tration of 0.7 %. Addition of the detergent, homogenization, and centri-

fugation were repeated as described in step 1. The resulting supernatant

(Fraction D) containing the 0.7 % NP-40 membrane extract was decanted.

This light brown, slightly opaque solution was used fresh for further

purification of the 5'-deiodinase or stored in capped vials in 10 mL ali-

quots at -60 °C. When stored in this manner, Fraction D could be kept

for up to 1 month with little or no loss of catalytic activity. The

remaining unsolubilized membrane (Fraction E) was discarded.

The quantity of detergent solution (B mL) added in the second step

was calculated from the following formula with the aid of a Texas Instru-

$$B = \frac{\left(\frac{c\%}{b\%}\right) \cdot A \; - \; \left(\frac{A \cdot a\%}{b\%}\right)}{1 \; - \; \left(\frac{c\%}{b\%}\right)}$$

ments TI-59 programmable calculator. The equation was derived from the

formula: (A mL · a%) + (B mL · b%) = (C mL · c%), with the assumption

that A + B = C. The terms are defined as follows: A = mL homogenate; B

= mL detergent to be added (unknown); and C = mL of final volume (un-

known). The small case letters (a, b, anc c) represent concentrations of

the extract already or to be added to each of the respective solutions.

Purification of Nonidet P-40 Solubilized Iodothyronine 5'-Deiodinase

Purification techniques attempted but not used in the procedure for

the purification of the Fraction D 5'-deiodinase included: salt frac-

tionation with ammonium sulfate (Weir, 1973), and fractionation with polyethylene glycol (Chesebro and Svehag, 1968). A 5-fold dilution of Fraction D, was used for cationic exchange cellulose chromatography with carboxymethyl (CM) cellulose (Wharton and McCarty, 1972), affinity chromatography with Affi-Gel 401 (Brocklehurst et al., 1974), and organomercurial-Sepharose 4B (Sluyterm and Wijdenes, 1974) and lectin binding chromatography with Concanavalin A-Sephrose 4B (Norden and O'Brian, 1974). These techniques were performed on Fraction D in buffer C as previously described, with minor modifications. All steps were carried out at 4 °C with the exception of Concanavalin A column elution which was performed at room temperature. All samples were stored at 4 °C or on ice prior to determination of enzyme catalytic activity. Concentrations of reagents used, and a description of the effectiveness of each technique are given in the Results section.

DEAE Cellulose chromatography was performed for the initial purification of the Fraction D preparation. DEAE Cellulose was prepared fresh or regenerated after previous usages by the method of Brewer (1974). A 2.4 x 14 cm column of cellulose, equilibrated in buffer D, having a bed volume of 55 mL was prepared at least 16 h prior to use. Fraction D in a 20-25 mL aliquot containing from 350 to 400 mg of protein was applied to the cellulose column at a flow rate of 40 mL/h. Following addition of the enzyme preparation, the column was washed with buffer D until the A_{280} dropped to base line. Typically, this required approximately 3 to 4 bed volumes of buffer D. Elution of the bound protein was performed in either a stepwise manner or with a linear gradient by increasing the concentration of salt in buffer D from 10 to 500 mmol/L NaCl. Individual

fractions were divided into 3 pools: Pool 1 contained the proteins that eluted with the sample front. Pool 2 (Fraction I) contained the majority of the 5'-deiodinase, and consisted of those fractions collected following the initial drop of A_{280}, but prior to the addition of NaCl. Pool 3 was the NaCl eluted protein.

Fraction I from the cellulose chromatography step was added in a ratio of 3.4 mg protein : 1 mL hydroxylapatite gel to a beaker and stirred slowly (to avoid foaming) for 16 h. Following the batchwise adsorption, the hydroxylapatite gel was recovered by centrifugation of the slurry at 1,000 x $\underline{g}$ for 10 min, and the recovered hydroxylapatite was poured into a 2.4 x 16 cm column for column chromatography. The column was washed with buffer F at a flow rate of 20 mL/h until the effluent was free of protein and then eluted with a step gradient of increasing KH_2PO_4 concentration. The step gradient was made by diluting buffer G with buffer F to give solutions of: 50, 125, and 400 mmol/L KH_2PO_4. Approximately 5 bed volumes of each elution buffer were applied to the column and the fractions collected at a concentration of 125 mmol/L phosphate were pooled together for the Fraction II enzyme preparation.

A Sephadex G-25 gel filtration column (2.6 x 90 cm) equilibrated in buffer F was used to desalt Fraction II. Sample volumes of up to 65 mL were applied to the 300 mL bed volume column at a flow rate of 70 mL/h, and the effluent fraction containing 5'-deiodinase activity were pooled and concentrated on a mini-column (3 mL bed volume) of hydroxylapatite equilibrated in buffer F. The desalted enzyme solution was applied at a flow rate of 12 mL/h, and the adsorbed protein was recovered with a 300 mmol/L phosphate elution buffer at a flow rate of 4 mL/h. This technique

allowed the partially purified enzyme to be recovered in a volume of approximately 6 mL.

A Sepharose 6B-CL gel filtration column (1.5 x 98.5 cm) was used for the third step in the purification sequence. Fraction II concentrate was applied to the column in 3 to 6 mL aliquots, and chromatography in buffer H was performed at a flow rate of 20 mL/h. Fractions were collected and assayed for thyroxine 5'-deiodinase activity. Those fractions containing high concentrations of activity were pooled and labeled as Fraction III. This fraction was stored in tightly closed vials at 4 °C prior to use in characterization studies.

Isoelectric Focusing

Isoelectric focusing of the Fraction III iodothyronine 5'-deiodinase was performed at 4 °C in a 110 mL electrofocusing column mounted in a vertical position. When the cathode was used at the bottom of the column the column was prepared in the following manner: 12.5 mL of the cathode buffer were added into the center chamber of the column. The LKB gradient mixer was used to develop the sucrose gradient as 55 mL of the heavy gradient solution and 55 mL of the light gradient solution, containing the protein sample were added to the column. Following the addition of the sucrose gradient, 12.5 mL of the anode buffer were added to the top of the gradient. Electrophoresis was begun, following the release of the cathode chamber plug, by adjusting the power source to deliver an initial output of 200 v. The voltage was gradually increased to 400 v keeping the wattage below 1.5 watts throughout the run. The voltage was increased to 350 v after 10 h and to 400 v after 19 h. The

column was developed an additional 26 h at 400 v. Prior to turning off the voltage, the amperage had stabilized to 1.4 ma for 3 h. The plug was used to close off the cathode chamber and the column was eluted.

The sucrose gradient was eluted at a rate of 2 mL/min with the aid of a peristaltic pump. The column effluent was collected in 1.5 mL fractions and immediately following the collection of the effluent, the individual fractions were covered with Parafilm and placed on ice. The pH of each fraction was measured with a digital pH meter standardized with buffers at 0-4 °C. The protein concentration and activity of the iodothyronine 5'-deiodinase were also determined for each fraction.

Reagents for Isoelectric Focusing:

Anode Buffer. 100 μL of 85 % phosphoric acid, 125 μL of 10 % Nonidet P-40, and 625 μL of 100 mmol/L dithiothreitol were diluted to 12.5 mL with deionized water.

Cathode Buffer. 5.725 g sucrose, 1.25 mL of 10% NaOH, 125 μL 10% Nonidet P-40, and 625 μL of 100 mmol/L dithiothreitol were diluted to 12.5 mL with deionized water.

Light Gradient Solution. 10 mL of protein sample (Fraction III), 1.25 mL of ampholytes (3/10), 0.6 mL of 10 % Nonidet P-40, and 3.0 mL of 100 mmol/L dithiothreitol were dissolved in deionized water and diluted to a final volume of 60 mL.

Heavy Gradient Solution. 28.0 g sucrose, 3.75 mL ampholytes (3/10), 0.6 mL 10 % Nonidet P-40, 3.0 mL of 100 mmol/L dithiothreitol were diluted to a final volume of 60 ml with deionized water.

Molecular Weight Determination

The apparent molecular weight of the detergent solubilized iodothyronine 5'-deiodinase was approximated by analytical gel filtration chromatography. A Sephacryl S-300 superfine column (1.5 x 98.4 cm) was calibrated with the Pharmacia low molecular weight and high molecular weight calibration kits according to manufacturers recommendations. The following protein standards were used: ribonuclease A (13,700 daltons), chymotrypsinogen A (25,000 daltons), ovalbumin (43,000 daltons), bovine serum albumin (67,000 daltons), aldolase (158,000 daltons), catalase (232,000 daltons), ferritin (440,000) daltons), and thyroglobulin (669,000 daltons). Blue dextran 2,000 was used to determine the void volume. Chromatography of both protein standards and enzyme preparations was performed in column buffer H (4 °C) at a rate of 12 mL/h. Gel filtration chromatography with the above standards was also performed on a Sephadex G-200 column (1.5 x 96 cm) and the Sepharose 6B-CL column (1.5 x 98 cm).

The approximate molecular weight of the iodothyronine 5'-deiodinase was determined from a plot of the log molecular weight versus the K_{av} of each standard. The K_{av} was determined from the equation $K_{av} = (V_e - V_o)/(V_t - V_o)$; where V_e is the elution volume for the protein, V_o is the void volume as determined by blue dextran, and V_t is the total bed volume of the gel. In addition to relating the elution volume of the enzyme to the protein standards to determine an approximate molecular weight for the enzyme, a plot of $-\log K_{av}$ versus the Stokes' radius was also used to approximate the molecular size of the solutilized enzyme.

Enzymatic Assays With ^{125}I-T4 and ^{125}I-rT3 Substrates

The 5'-deiodination of ^{125}I-labeled T4 and rT3 by the Fraction D and Fraction III enzyme preparations was carried out under standard enzyme assay conditions with the exception that the pH for the deiodination of rT3 was increased to pH 7.5 and the addition of radiolabeled substrates were added to the incubation mixture. The T4 and rT3 substrate solutions contained 6.5 μg T4 + 0.0572 μCi ^{125}I-T4/100 μL and 5.0 μg rT3 + 0.0092 μCi ^{125}I-rT3/100 μL, respectively. Prior to use, the radiolabeled substrates were purified by Sephadex G-25 column chromatography according to the method of Green (1972). The reaction was stopped with the addition of 50 μL of a solution containing 2 mol/L NaOH and 5 mol/L NaCl rather than 1.0 mL of the reagent alcohol. This resulted in a final pH of 12.5 or higher. The percent ^{125}I-T4 and ^{125}I-rT3 deiodinated was determined by separating the substrates and products of the reaction mixture by Sephadex G-25 column chromatography (Green, 1972; Burman et al., 1977) and counting the radioactivity in each of the peaks.

Sephadex G-25 chromatography was performed in 0.8 x 19 cm columns (10.0 mL bed volume) at a flow rate of approximately 20 mL/h. The sample was added in a solution having a final concentration of 0.015 mol/L NaOH, 0.5 mol/L NaCl. The column buffer was 0.1 mol/L NaOH, 0.005 mol/L NaCl. A sample volume of 400 μL was applied and fraction volumes of 1.03 mL were collected. The void volume was 5.0 mL. Free iodide (^{125}I) eluted in a sharp peak (8-12 mL) with an elution volume V_e of 10.0 mL. ^{125}I-T3 and the rT3 metabolite (3,3'-T2) eluted in a sharp peak (15 to 20 mL) with a V_e of 17.0 mL. ^{125}I-T4 and ^{125}I-rT3 eluted in a broad peak (22-30 mL) with a V_e of 25.5 mL. A single column run required approximately 90

min to complete. The columns demonstrated excellent reproducibility and

sample recovery was essentially complete.

CHAPTER III

RESULTS

Subcellular Fractionation

The subcellular distribution of iodothyronine 5'-deiodinase in rat

liver cells was studied by differential centrifugation as described in

METHODS. The results are presented in Table 2. Deiodinase activity was

found in all fractions studied. The fraction with the least amount of

activity was the 78,000 x g supernatant. Of the particulate enzyme

fractions, the greatest concentrations of 5'-deiodinase activity were

found in the crude microsomal and crude nuclear fractions.

The crude microsomal fraction (Fraction P) and the crude nuclear

fraction (Fraction N) were further subdivided by ultracentrifugation in a

discountinuous sucrose gradient. When the resulting plasma membrane sub-

fractions were assayed for 5'-deiodinase activity, it was found that the

deiodinase was distributed throughout the membrane fractions rather than

being predominately associated with any one fraction. The subfraction

with the greatest yield of deiodinase activity was the microsomal en-

riched fraction (Fraction P4) which had a 30 % yield of 5'-deiodinase

activity and a 2.5-fold increase in specific activity. An additional 38

% of the deiodinase activity was recovered in Fractions: N2, N3, P2 and

P3, as shown in Table 2. The subfractions of greatest specific activity

were the plasma membrane fractions (N2) and (P2) and the endoplasmic

reticulum enriched plasma membrane fraction (P3), which had relative

specific activities of 6.1, 6.8, and 10.1, respectively.

Table 2

Distribution of Iodothyronine 5'-Deiodinase Activity Following

Subcellular Fractionation of Rat Liver Homogenate

FRACTION	PROTEIN (% of total)	5'-DEIODINASE ACTIVITY (% of total)	RSA*	(n)**
(N) Nuclei (1,000 x g pellet)	17.6 ± 4.3	30.5 ± 6.0	1.73 ± 0.62	6
(M + L) Mitochondria plus Lysosomes (33,000 x g pellet)	15.2 ± 2.8	13.4 ± 4.1	0.88 ± 0.28	6
(P) Microsomes (78,000 x g pellet)	14.1 ± 2.8	48.2 ± 8.7	3.42 ± 1.03	6
(S) Cytosol (78,000 x g supernate)	48.0 ± 3.7	6.8 ± 3.1	0.14 ± 0.03	9
Recovery From Homogenate:	94.9 ± 8	98.9 ± 14		
(N2) Plasma Membrane (pm)	1.7	10.3	6.1	2
(N3) pm + Mitochondria	7.6	9.1	1.2	2
(N4) Nuclei	8.2	8.8	1.1	2
Recovery From Fraction N:	17.5	28.2		
(P2) Plasma Membrane	1.2	8.2	6.8	2
(P3) pm + Endoplasmic Reticulum	1.0	10.1	10.1	2
(P4) pm + Microsomes	11.9	29.5	2.5	2
Recovery From Fraction P:	14.1	47.8		

Data are mean ± standard deviation, or the average of duplicate determinations.

* RSA = Relative Specific Activity (RSA of the crude homogenate = 1.0).
** (n) = number of fractionation runs.

Solubilization of Iodothyronine 5'-Deiodinase

Results from the subcellular fractionation study indicated that the iodothyronine 5'-deiodinase was primarily a particulate enzyme and, therefore, that membrane extraction would be required prior to purification. A crude membrane preparation (Fraction C) was used as the starting material and several techniques for the solubilization of the 5'-deiodinase were evaluated.

Two physical methods were attempted to extract the enzyme. Extensive membrane homogenization with Potter-Elvehjem or Dounce homogenizers did not increase the yield of soluble 5'-deiodinase activity significantly. When Fraction C was subjected to 8 freeze-thaw cycles, 10 % or less of the total particulate activity was extracted.

A number of chemical extraction techniques were also investigated. Acid extraction failed to solubilize the 5'-deiodinase and at pH's below 5.8 resulted in a sharp decrease in recoverable particulate activity. Alkali treatment also failed to extract the enzyme, and resulted in a more gradual decrease in the recovery of particulate activity. This decrease became more prominent above pH 7.8. Neither n-butanol nor ethanol were effective in extracting active enzyme from Fraction C when solvent concentrations of from 0.1 % to 5.0 % were tested. The enzyme was completely inactivated by extraction at 4 °C with concentrations of n-butanol greater than 4.0 %. The inactivation of the enzyme during ethanol extraction at 4 °C was less severe, but still approached 50 % at an ethanol concentration of 5.0 %.

Enzymatic digestion of Fraction C with pancreatic lipase (0.02-0.16 % did not extract protein or 5'-deiodinase activity. At a lipase

concentration of 0.32 %, approximately 20 % of the protein content of Fraction C was released. Higher concentrations of lipase released greater amounts of protein, however, 5'-deiodinase activity was not observed in the lipase extracts at any of the concentrations tested.

Detergent solubilization of the crude membrane preparation with the anionic detergent, deoxycholate (DOC); the nonionic detergent, Triton X-100 (TX-100); Nonidet P-40 (NP-40); and zwitterionic detergents (Zwittergents: 3-08, 3-10, 3-12, 3-14, and 3-16) was also evaluated. The solubilization of the crude membrane preparation to clarity with the dropwise addition of a 10 % solution of DOC resulted in a complete loss of deiodinase activity. A partial reactivation of the 5'-deiodinase could be achieved by removing DOC via Sephadex G-25 filtration, however, the yield of solubilized enzyme activity from Fraction C was less than 16 % using this technique. When DOC concentrations of 0.18, 0.30, 0.60, and 0.80 % were used the yields of solubilized activity were: 18, 24, 8, and 2 %, respectively (assayed in the presence of DOC). The solubilization of Fraction C with several nonionic and zwitterionic detergents at final detergent concentrations of 0.3 % gave the following results when enzyme assays were carried out in the presence of the solubilizing detergent: Triton X-100 solubilized 64 % of the protein and 30 % of the deiodinase activity, NP-40 solubilized 68 % of the protein and 41 % of the deiodinase activity, and the Zwittergents: 3-08, 3-10, 3-12, 3-14, and 3-16 solubilized 35, 55, 70, 80, and 53 % of the protein respectively and 4, 22, 28, 14, and 16 % of the deiodinase activity respectively from Fraction C. Further experiments using combinations of DOC and NP-40, and combinations of the Zwittergents and NP-40 did not increase the yield of

solubilized 5'-deiodinase.

Based on the results of the extraction techniques presented above, detergent solubilization with NP-40 was chosen as the most suitable method for the extraction of active iodothyronine 5'-deiodinase from crude rat liver membrane preparations. To determine the conditions necessary to selectively solubilize the 5'-deiodinase, further experiments were conducted to study the effect of detergent concentrations on membrane solubilization. The results are presented in Figures 6 and 7. Homogenization of Fraction C with 0.05 % NP-40 solubilized 55 % of the protein and 4.6 % of the 5'-deiodinase activity. Protein solubilization reached a plateau above 0.4 % detergent and solubilization with 0.7 % detergent extracted 78 % of the protein from Fraction C and resulted in a 60 % yield of solubilized 5'-deiodinase activity. The apparent inactivation of the total 5'-deiodinase activity in the presence of increasing concentrations of detergent (Figure 7) could not be reversed by detergent removal and, therefore, limited the maximum yield of solubilized enzyme activity to approximately 60 % of that present in Fraction C.

Other experiments demonstrated that the yield of active 5'-deiodinase was dependent on the pH of the extraction buffer. The optimum pH for solubilization with NP-40 was found to be in the range of pH 7.0 to 7.5.

Purification of Iodothyronine 5'-Deiodinase

Preliminary experiments were performed to evaluate several purification techniques as to their potential usefulness in the purification of the crude NP-40 solubilized deiodinase (Fraction D). These techniques

Figure 6. Effect of Nonidet P-40 concentration on the solubilization of protein from rat liver membranes.

        Varying quantities of detergent were added (4 °C) in a dropwise manner with stirring to a sample of Fraction C containing 65 mg of protein. The final volume was adjusted to 10 mL with homogenate buffer (pH 7.35), and the mixture was homogenized in the Dounce homogenizer with 12 strokes of pestle A. The soluble protein was separated from the pellet following centrifugation (37,000 x $\underline{g}$, 45 min). The solubilized protein (O), unsolubilized protein (△) and total protein (●) are plotted as a function of Nonidet P-40 concentration. Data are the averages of duplicate determinations.
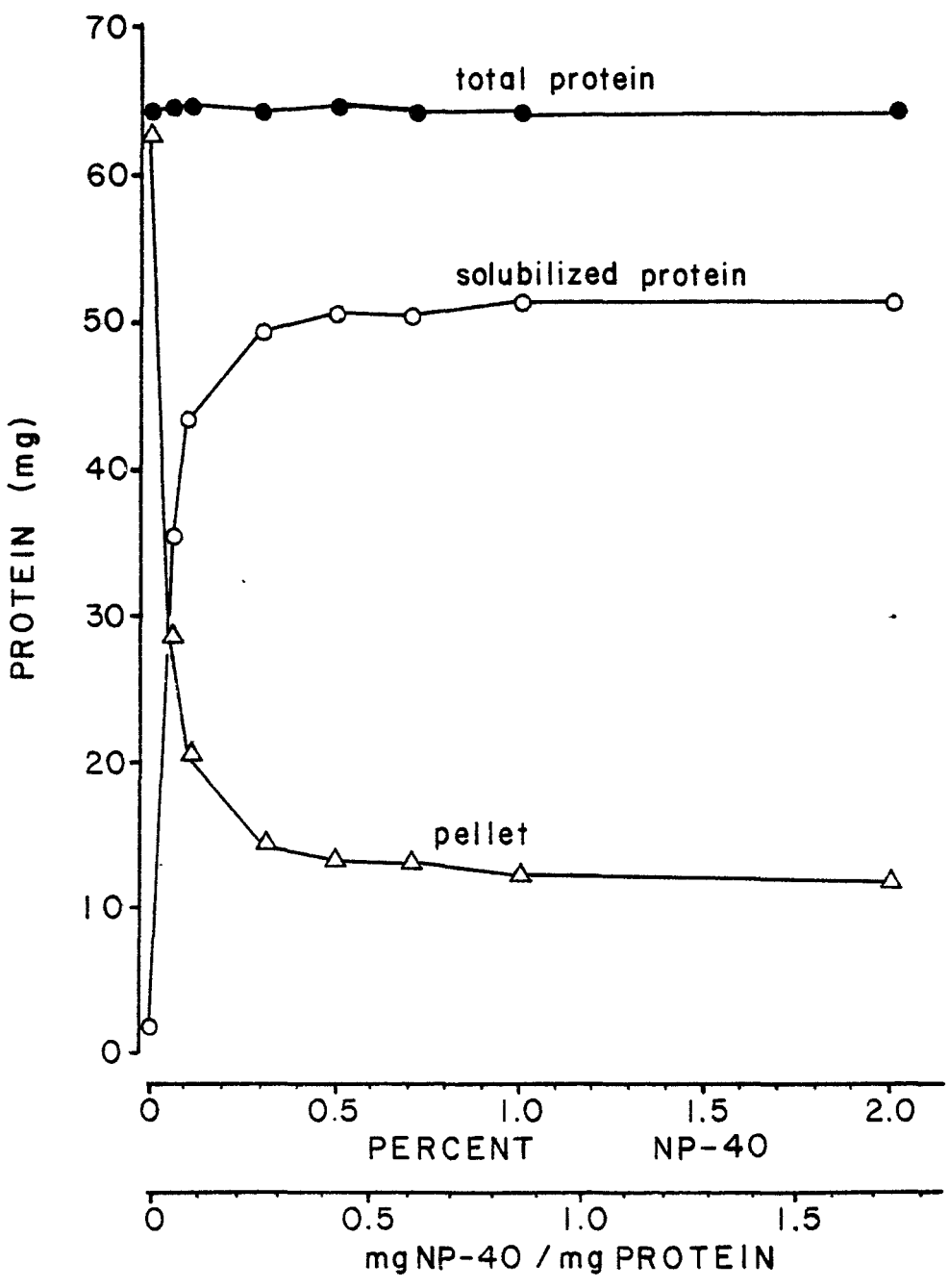
Figure 7.   Effect of Nonidet P-40 concentration on the solubilization of iodothyronine 5'-deiodinase activity from rat liver membranes.

Varying quantities of detergent were added (4 °C) in a dropwise manner with stirring to a sample of Fraction C containing 65 mg of protein.   The final volume was adjusted to 10 mL with homogenate buffer (pH 7.35), and the mixture was homogenized in the Dounce homogenizer with 12 strokes of pestle A.   The solubilized proteins were separated from the pellet following centrifugation (37,000 x g, 45 min).   T4 to T3 converting activity in the solubilized extracts (O) and in the pellet (△) was determined by standard 5'-deiodinase assay conditions (See MATERIALS AND METHODS).   The detergent concentration for all samples was adjusted to 0.1 % NP-40 prior to enzyme assay.   Total activity (●) is the sum of solubilized and pellet activities.   Data are the averages of duplicate determinations.
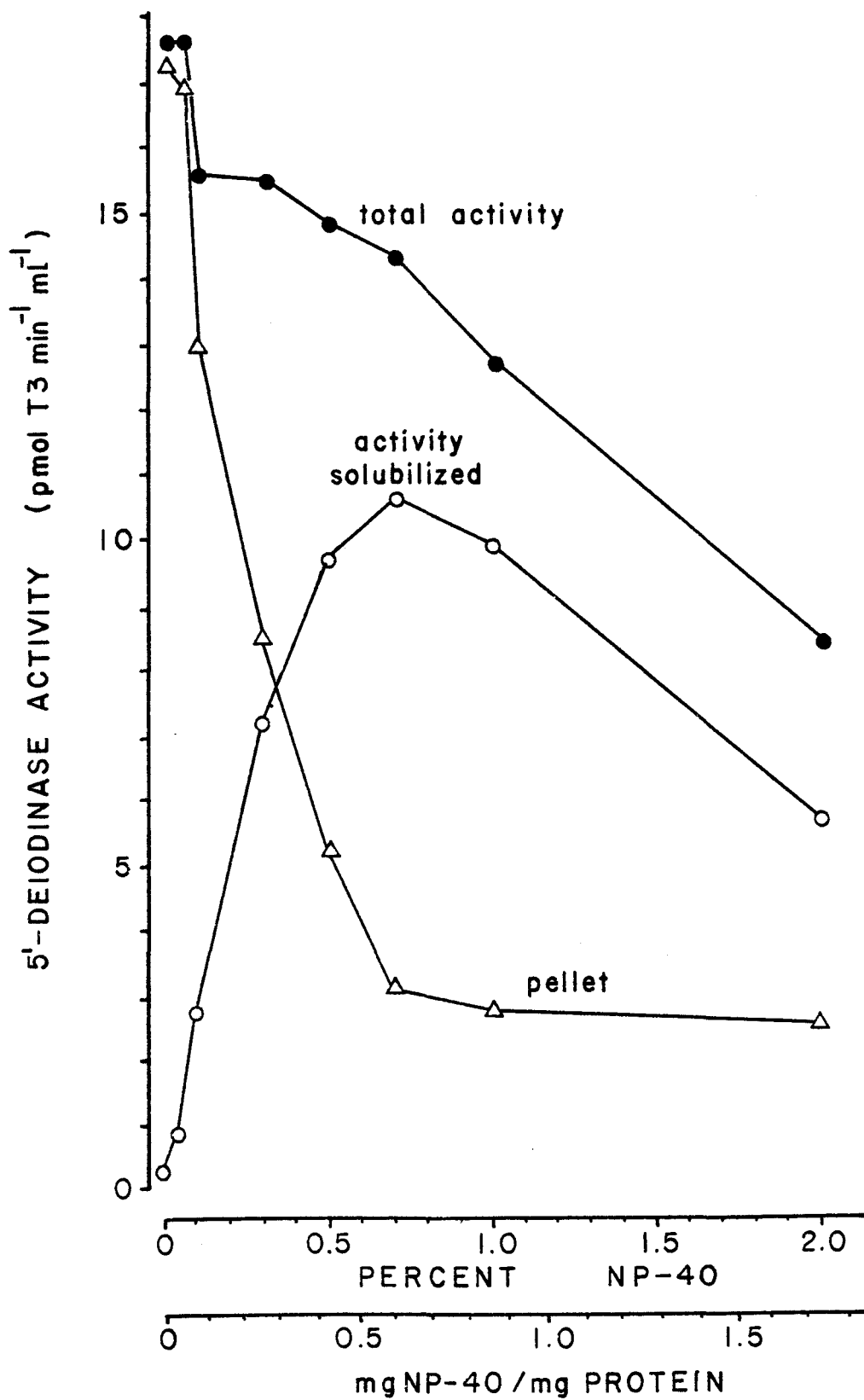
included affinity chromatography, ion exchange chromatography, chromatography using hydroxylapatite, gel filtration chromatography and fractionation with ammonium sulfate and polyethylene glycol. Additional experiments were also performed to evaluate methods for desalting and concentrating solutions of the enzyme.

When the crude NP-40 solubilized preparation was applied to a column of Concanavalin A-Sepharose 4B, 88 % of the 5'-deiodinase and 93 % of the protein passed through the column unadsorbed. Elution of the column with 750 mmol/L alpha-methyl-mannoside resulted in the recovery of the remaining 7 % protein, but no additional 5'-deiodinase activity. The total recovery of 5'-deiodinase was 88 %. In experiments using an Affi·Gel 401 sulfhydryl-binding affinity column, an average 72 % of the applied enzyme and 97 % of the applied protein was recovered in the unbound fraction. An additional 3-8 % of the 5'-deiodinase and the remaining 3 % protein was recovered by elution with 50 mmol/L L-cysteine. The total recovery of 5'-deiodinase activity from the Affi·Gel column was approximately 77 %. When an organomercurial Sepharose 4B resin was used as a sulfhydryl-binding adsorbent, approximately 18 % of the applied deiodinase and 56 % of the applied protein did not bind to the column. When the column was eluted with 50 mmol/L L-cysteine, 25 % of the 5'-deiodinase activity and 44 % of the protein were recovered. The total recovery of 5'-deiodinase activity was 43 %. A similar yield of enzyme activity and protein were obtained when the organomercurial column was eluted with 25 mmol/L dithiothreitol.

Both cation and anion exchange celluloses were used to evaluate ion exchange chromatography of the crude NP-40 solubilized 5'-deiodinase.

During cation exchange chromatography of Fraction D on CM cellulose equilibrated to pH 6.4, the unadsorbed protein eluted from the column in an initial sharp peak containing 64 % of the deiodinase activity and 70 % of the protein. A second smaller peak of unadsorbed protein trailed behind the first and contained an additional 18 % of the deiodinase and 22 % of the protein from the applied sample. Elution of the adsorbed protein with 350 mmol/L NaCl resulted in the recovery of 6 % of the enzyme and 8 % of the protein. The total recovery of protein was 98 %, and the total recovery of enzyme activity was 88 %. Elution with increased concentrations of NaCl failed to recover additional activity from the column. Chromatography at pH's less than 6.4 resulted in decreased recoveries of enzyme activity due to the tendency of the detergent solubilized 5'-deiodinase to precipitate under acidic conditions.

Anion exchange chromatography was performed using DEAE cellulose. Maximum recovery of enzyme activity occured when the column and sample were equilibrated to pH 7.6. The majority of the unadsorbed protein eluted from the DEAE cellulose column in a sharp peak containing approximately 20-30 % of the applied protein and 10-30 % of the applied deiodinase. Following the initial peak of unadsorbed protein, small amounts of protein eluted as the column was washed with buffer. This trailing peak contained 8-15 % of the applied protein and 50-65 % of the applied 5'-deiodinase activity. In a typical run, 20 % of the applied enzyme and 65 % of the applied protein adsorbed to the column matrix. When a linear gradient of increasing NaCl concentration was then applied to the column, additional 5'-deiodinase activity eluted from the column in amounts proportional to the peaks and troughs of the total protein elution pattern.

The largest peak of 5'-deiodinase activity was eluted at a NaCl concentration of 100 mmol/L. The specific activity of this peak was calculated to be 0.12 relative to 1.0 for the crude Fraction A, and 1.29 for the applied sample. In a typical run, the total recovery of protein was approximately 100 %, and the total recovery of deiodinase activity was in the range of from 120-130 %, suggesting that a mild activation of the 5'-deiodinase occured during the DEAE cellulose step.

Further experiments with DEAE cellulose demonstrated that the 5'-deiodinase had a low affinity for the diethylaminoethyl ligand of the cellulose matrix at pH 7.6. When a previously adsorbed peak was pooled, desalted, and reapplied to DEAE cellulose, 43 % of the enzyme and 86 % of the protein readsorbed. The remainder of the sample eluted in a single broad peak with the low ionic strength column buffer. When the unadsorbed activity was pooled and reapplied, an additional 4 % of the enzyme and 9 % of the protein adsorbed to the DEAE cellulose.

Addition of the crude NP-40 solubilized membrane extract to a hydroxylapatite column resulted in the adsorption of 94 % of the applied enzyme and 72 % of the applied protein. Elution of the adsorbed enzyme from the hydroxylapatite column with a linear gradient of increasing phospage concentration, resulted in a broad wavy peak of 5'-deiodinase activity which coincided with the bulk of the eluted protein. In a similar manner, elution of the enzyme from the hydroxylapatite column with a step gradient resulted in elution of enzyme activity over a broad range of phosphate concentrations in such a manner that there was no significant increase in specific activity for any of the peaks obtained. A single sharp peak of activity could be recovered from the hydroxylapatite

column, however, when the applied sample had been previously purified by DEAE cellulose chromatography. Elution of the partially purified 5'-deiodinase from the hydroxylapatite column with a linear phosphate gradient resulted in the recovery of 76 % of the applied enzyme within a concentration range of 90 to 150 mmol/L phosphate. Elution of the partially purified enzyme with a step gradient of: 100, 150, 200 and 300 mmol/L phosphate resulted in the recovery of: 49.3, 19.9, 11.3, and 15.5 %, respectively of the applied enzyme activity and 11.1, 16.9, 24.4, and 21.0 %, respectively of the applied protein. The 100 mmol/L phosphate step resulted in a 4.4-fold increase in specific activity. Approximately 24 % of the protein and 4 % of the enzyme did not adsorb to the hydroxylapatite column.

Gel filtration chromatography of the crude NP-40 solubilized membrane extract was performed on columns of Sephadex G-100 and Sepharose 6B-CL. When the detergent extract was added to Sephadex G-100, and chromatographed in the presence of detergent, 98 % of the deiodinase activity and 42 % of the protein from the sample eluted in the void volume. The included volume contained 58 % of the sample protein and no deiodinase activity. When the detergent extract was applied to Sepharose 6B-CL, 6 % of the deiodinase eluted in the void volume, 11 % eluted as a large molecular weight complex in excess of 400,000, and 54 % was recovered in a symmetrical peak corresponding to an average molecular weight of 280,000. The total recovery of 5'-deiodinase activity was 71 %. The protein eluted in skewed fashion towards the lower molecular weight range and the purification of the major activity peak was estimated to be 2.4-fold. Gel filtration was normally performed with a column buffer containing

0.1 % NP-40 detergent. In the absence of detergent, the 5'-deiodinase eluted from the Sepharose 6B-CL column in the void volume.

Fractionation of the NP-40 membrane extract with ammonium sulfate and polyethylene glycol resulted in yields of activity in the range of 30 to 50 %. The losses of enzymatic activity appeared to be due to the incomplete resolubilization of the precipitated protein. Ammonium sulfate precipitated 80 % of the 5'-deiodinase activity at 45 % saturation (4 °C) with a resulting 1.8-fold purification. Fractionation with polyethylene glycol at concentrations of from 10-12 % precipitated 80 % of the activity, however, due to the loss in recoverable activity and a near complete precipitation of protein, a decrease rather than an increase in specific activity was observed.

Desalting of 5'-deiodinase solutions was performed either by dialysis or by Sephadex G-25 gel filtration. Dialysis overnight in hydroxylapatite column buffer at 4 °C resulted in a 30-50 % loss in activity compared to a 0-10 % loss in activity using the Sephadex G-25 column. An additional advantage of using Sephadex G-25 chromatography was that it took less time.

When the NP-40 solubilized 5'-deiodinase was concentrated with an Amicon concentrator using a PM-30 membrane (30,000 molecular weight cutoff) at 4 °C, a 10-fold concentration was accompanied by a 90 % loss of activity. When mini-columns of hydroxylapatite were used for concentration, approximately 5 mg of protein was adsorbed per mL of hydroxylapatite resin and the 5'-deiodinase activity was recovered by eluting the columns with small volumes of 350 mmol/L phosphate elution buffer. The recovery of activity by this method was approximately 100 %.

The Purification Procedure

A multistep purification procedure (Figure 8) was developed based on detergent solubilization with Nonidet P-40 and the combined use of three separation techniques that gave promising results in the pilot studies described above. Each technique was optimized using the purified product from the previous step. Twelve different preparations were carried through the procedure. The results described below, for a single purification attempt, are characteristic of my experience with each step, and the overall purification scheme.

Preparation of Solubilized Iodothyronine 5'-Deiodinase

The crude rat liver homogenate (Fraction A) was prepared for solubilization as described in the Materials and Methods section. An initial high speed centrifugation step was used to remove the cell cytosol (Fraction B). The remaining membrane pellet (Fraction C) retained 92 % of the 5'-deiodinase activity and 53 % of the protein from Fraction A. This initial purification of the crude homogenate resulted in a 1.7-fold increase in specific activity. Following the removal of the cell cytosol, a 2 step selective solubilization of Fraction C was performed with Nonidet P-40. In the first step, Fraction C was homogenized with 0.05 % NP-40. This step solubilized 53 % of the remaining protein and 5 % of the 5'-deiodinase. The extract (Fraction C_2) was discarded. In the second step, the washed membrane pellet (Fraction C_1) was homogenized with a final 0.7 % NP-40. Approximately 60 % of the activity was solubilized membrane fraction (Fraction E). The use of 0.7 % detergent resulted in a 20 % loss in total activity from Fraction C_1. The recovery of total

Figure 8. Flow diagram for the solubilization and purification of iodothyronine 5'-deiodinase from rat liver homogenate.
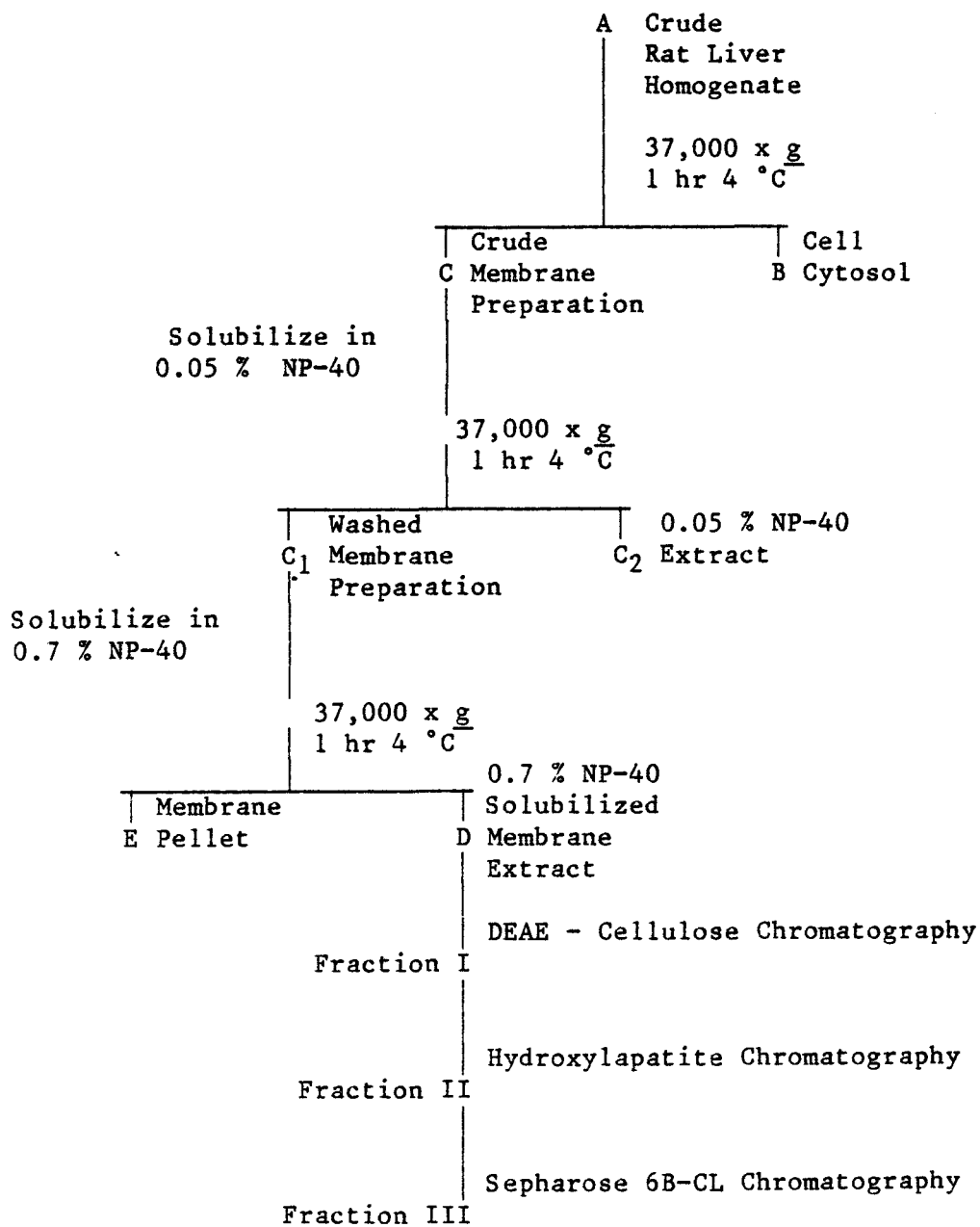
protein and total 5'-deiodinase activity from Fraction A is shown in Table 3.

The solubilized membrane extract (Fraction D) contained 20 % of the protein and 49 % of the activity of Fraction A, corresponding to a 2.4-fold increase in specific activity. Upon visual inspection, Fraction D was a slightly viscous, dark brown, turbid solution. The 5'-deiodinase activity did not sediment when centrifuged for 60 min at 77,000 x $\underline{g}$, however, the activity was rapidly precipitated when the pH of Fraction D was adjusted to pH 5.8 or below. Fraction D could be stored frozen at -60 °C for up to 6 weeks without a detectable loss in 5'-deiodinase activity.

DEAE Cellulose Chromatography

DEAE cellulose chromatography was used as the first step in the purification of Fraction D. The elution profile of protein and 5'-deiodinase activity from the DEAE cellulose column is shown in Figure 9. The optimum performance of this step occured at pH 7.6. The flow rate was not critical to the performance of the column as long as the sample front migrated evenly. The packing of the column, however, was a major factor. For optimum performance, the cellulose resin was prepared in large batches which were equilibrated in column buffer, and the columns were poured at least 16 h prior to use (See MATERIALS AND METHODS). A sample volume of up to 40 % of the bed volume could be applied and as much as 12 mg protein were adsorbed per milliliter of cellulose.

Approximately 65 % of the protein content of Fraction D and 23 % of the 5'-deiodinase activity adsorbed to the cellulose resin. Of the 35 %

Table 3

Preparation of Nonidet P-40 Solubilized

Iodothyronine 5'-Deiodinase

FRACTION*	NONIDET P-40 (g/100 mL)	PROTEIN (% YIELD)	5'-DEIODINASE ACTIVITY (% YIELD)
A**	--	100	100
B	--	47	8
C	--	53	92
C_1	0.05	25	85
C_2	0.05	28	5
D	0.70	20	49
E	0.70	5	19

* See Figure 8 for flow diagram of solubilization.
** Fraction A from 70 g of rat liver tissue contained 7.08 g of protein and 3,737 units (pmol T3 $min^{-1}mg^{-1}$) of iodothyronine 5'-deiodinase activity.

Figure 9. DEAE cellulose column chromatography of Fraction D.

        120 mL of Fraction D was applied in 30 mL portions to 4 separate
columns containing 2.4 x 16 cm beds (68 mL) of DEAE cellulose. Chromato-
graphy in each of the 4 columns was performed (4 °C) at a rate of 40
mL/h. Following elution of the bulk of unadsorbed protein (0-100 mL),
Fraction I, as indicated by the bar (⊢——⊣), was collected with an addi-
tional 3 bed volumes (205 mL) of column buffer. At the arrow (↓), 500
mmol/L NaCl was added to the column buffer to elute the adsorbed pro-
tein.
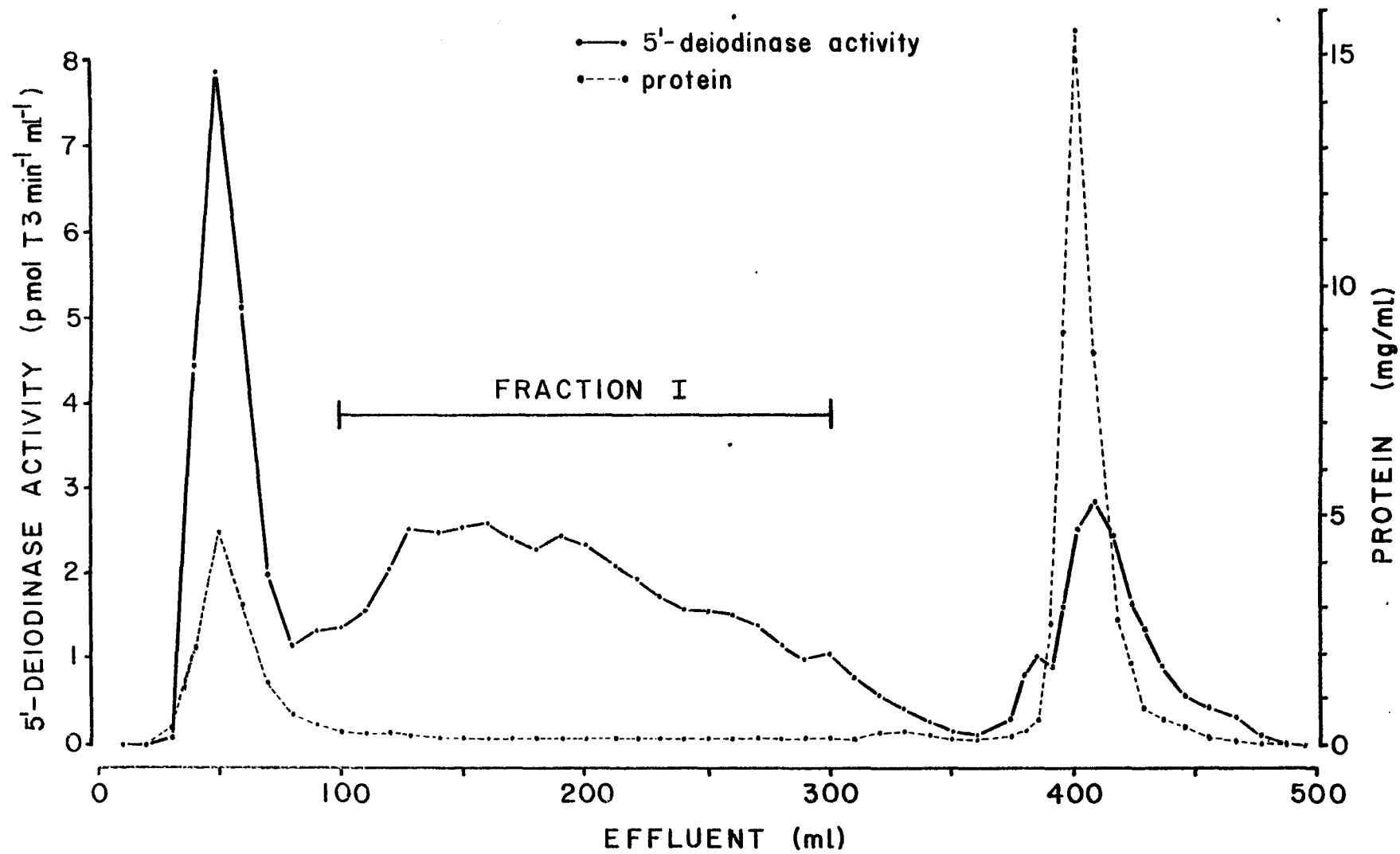
FRACTION I

protein that did not adsorb, 26 % eluted in the first 80 mL of effluent along with 26 % of the 5'-deiodinase activity and the remaining 9 % eluted when an additional 205 mL of column buffer was applied. This intermediate peak (Fraction I) contained 77 % of the recovered 5'-deiodinase activity. The total recovery of protein was approximately 100 % and the total recovery of enzymatic activity was approximately 127 %. The DEAE cellulose step resulted in a 9-fold purification of Fraction D. In comparison to the crude rat liver homogenate (Fraction A), Fraction I contained 1.7 % and 38 % of the original protein and 5'-deiodinase activity respectively, corresponding to a net 22-fold increase in specific activity.

Hydroxylapatite Chromatography

Due to the large volume of Fraction I (820 mL) and the slow flow rate (20 mL/h) of the hydroxylapatite column, Fraction I was applied to the hydroxylapatite gel by batch adsorption. The quantity of gel added was adjusted so that there would be no more than 4.0 mg protein/mL hydroxylapatite. The gel slurry was subsequently recovered by low speed centrifugation and packed into a column. The enzyme was eluted with a step gradient of increasing phosphate concentration (See Figure 10).

Approximately 16 % of the protein from Fraction I did not adsorb to the hydroxylapatite gel. There was no detectable unadsorbed enzyme activity. Elution of the adsorbed protein was carried out in 3 steps: The 50 mmol/L phosphate buffer eluted 8 % of the protein and 11 % of the deiodinase. Fraction II (Figure 10) was recovered with the 130 mmol/L phosphate elution buffer. This fraction contained 18 % of the applied

Figure 10. Hydroxylapatite column chromatography of Fraction I

The combined Fraction I effluent (820 mL) from the DEAE cellulose chromatography step was applied to 32 mL of hydroxylapatite gel in a 1 liter vessel prior to column chromatography. Details of the procedure are described in Materials and Methods. The supernatant, containing the unadsorbed protein was recovered following low speed centrifugation (10 min, 1,000 x g). The gel, containing the adsorbed protein was packed into a column, and the resulting hydroxylapatite bed (2.4 x 9 cm) was washed with 32 mL of column buffer. Elution of the adsorbed protein was carried out at a flow rate of 20 mL/h (4 °C) with a step gradient of increasing phosphate concentration. At the points indicated by the arrows (↓), elution buffers containing: (A) 50 mmol/L, (B) 130 mmol/L and (C) 400 mmol/L phosphate were added to the column. The 130 mmol/L phosphate peak (Fraction II), as indicated by the bar (⊢——⊣), was pooled for gel filtration chromatography.
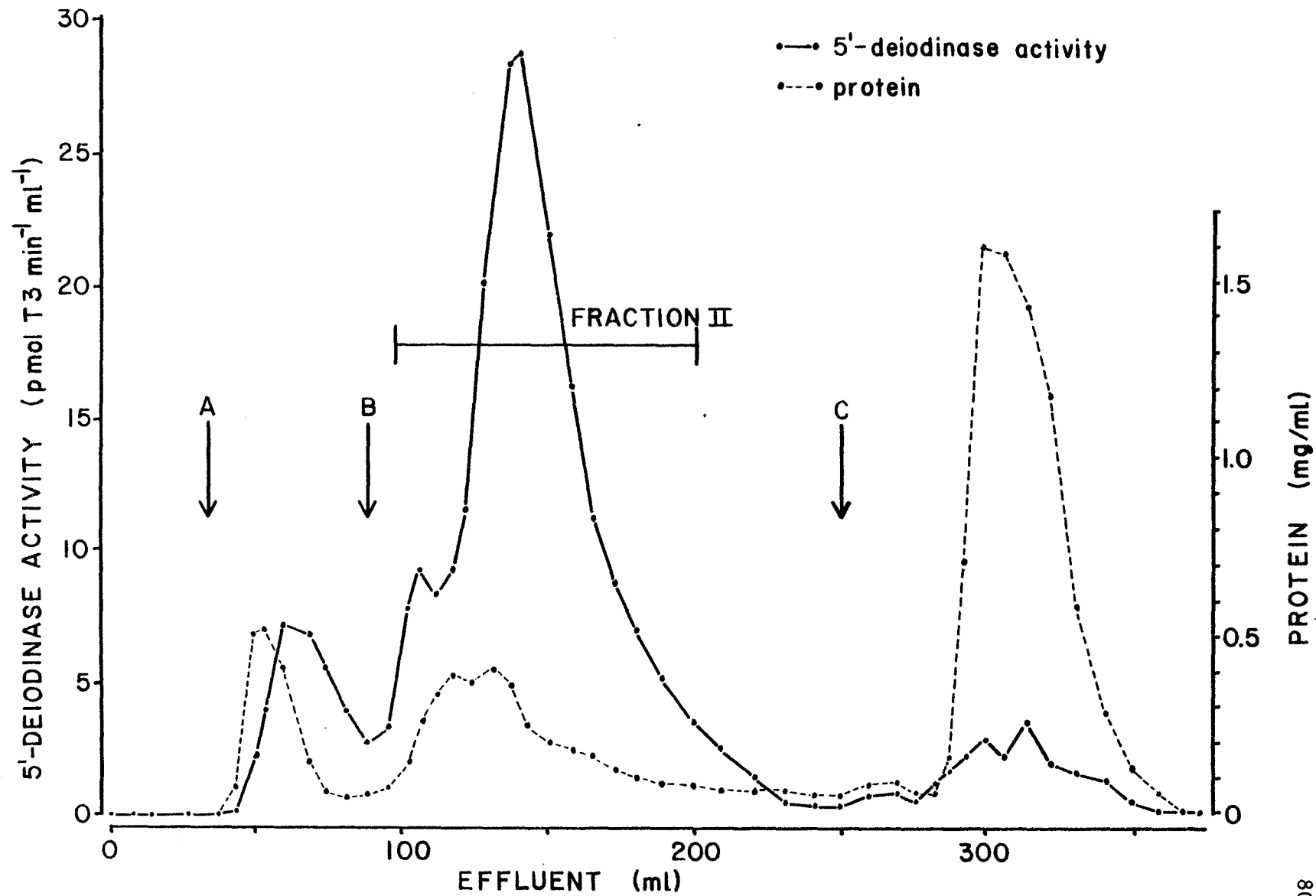
protein and 97 % of the applied 5'-deiodinase. The final 400 mmol/L
phosphate step eluted 57 % of the protein and 5 % of the 5'-deiodinase.
The recovery of protein was nearly complete and the net recovery of 5'-
deiodinase activity was 113 %. Fraction II was purified 5.3-fold with
respect to Fraction I and 115-fold relative to the crude rat liver homo-
genate (Fraction A). A final 37 % yield of 5'-deiodinase activity from
Fraction A was achieved.

Sepharose 6B-CL Chromatography

Sepharose 6B-CL column chromatography of Fraction II was used for
the final step in the purification of the iodothyronine 5'-deiodinase
(Figure 11). Optimal performance of the gel filtration step was observed·
when the column was run at a flow rate of 20 mL/h and when the column
buffer contained 0.1 % NP-40. The majority of the 5'-deiodinase activity
eluted from the column in a single sharp peak in the molecular weight
range of 200,000 -350,000. This peak (Fraction III) contained approxi-
mately 13 % of the protein and 66 % of the 5'-deiodinase activity reco-
vered from Fraction II. No activity and very little protein was detected
in the void volume, however, an additional 7 % of the deiodinase was re-
covered in 2 small peaks in the high molecular weight range and in a
trailing of the major activity peak towards the low molecular weight
range. The total recovery of enzymatic activity from Fraction II was
approximately 73 %. The gel filtration step increased the specific
activity 5.1-fold.

The final preparation (Fraction III) was purified 580-fold relative
to the crude rat liver homogenate. The overall recovery of activity was

Figure 11. Sepharose 6B-CL column chromatography of Fraction II.

Fraction II from hydroxylapatite chromatography was concentrated to 6 mL and applied to a column of Sepharose 6B-CL (1.5 x 98.5 cm). Gel filtration chromatography was performed at a rate of 20 mL/h (4 °C). The bar (⊢——⊣) indicates those fractions pooled for Fraction III. a-e indicate the peak volumes for blue dextran and the standard proteins used to calibrate the column: a, blue dextran; b, thyroglobulin; c, ferritin; d, catalase; e, aldolase.
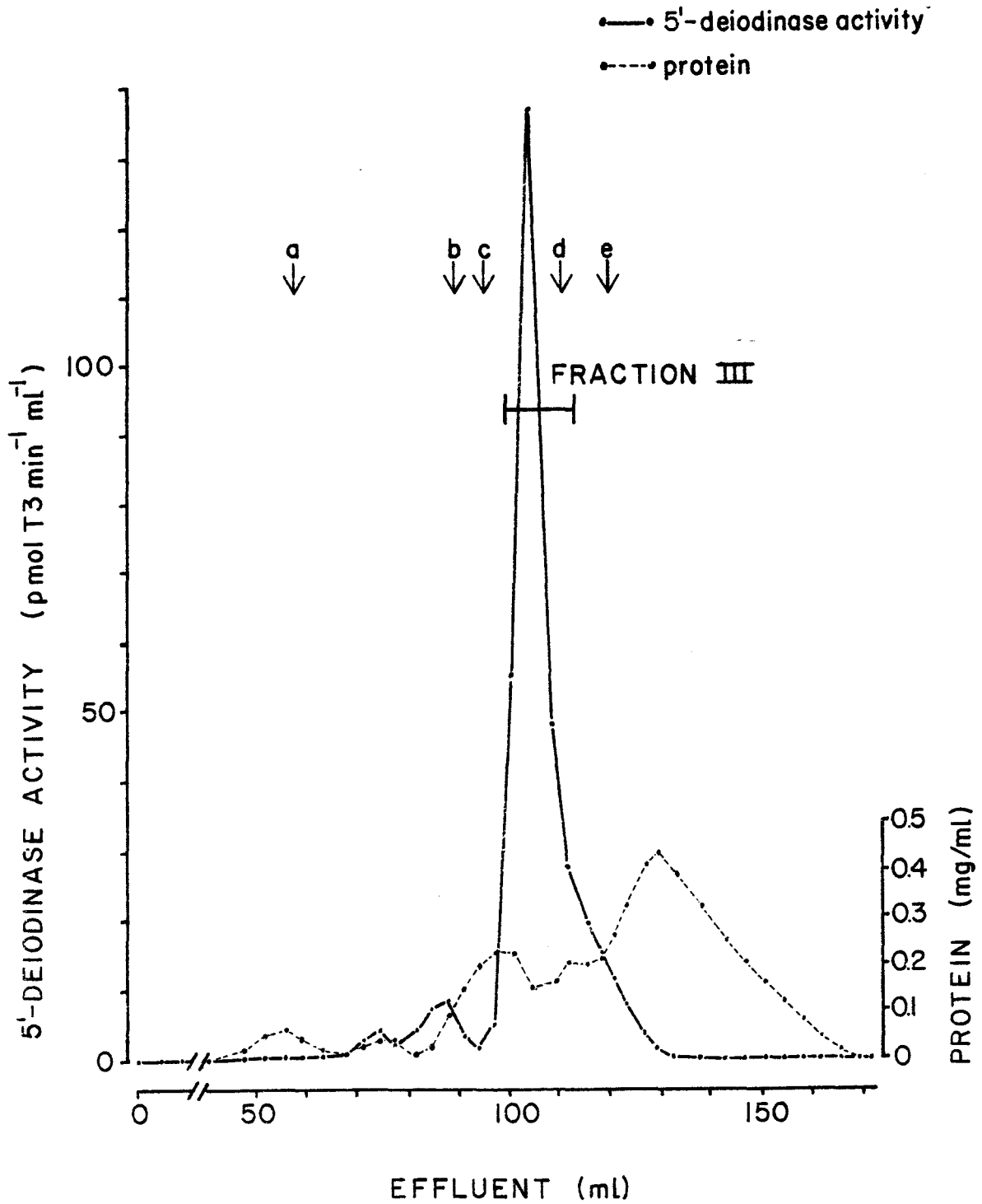
Table 4

Purification of Iodothyronine 5'-Deiodinase

From 70 Grams of Rat Liver

Fraction	Preparation	Volume (mL)	Total Protein (mg)	Enzyme* Concentration (units/mL)	Specific* Activity (units/mg)	Total* Units of Activity	Recovery (%)	Purification Factor
A	Crude Rat Liver Homogenate	250	7082	14.9	0.53	3737	100	1.0
C	Crude Membrane Preparation	210	3753	16.4	0.92	3451	92	1.7
D	Solubilized Membrane	120	1416	15.2	1.29	1823	39	2.4
I	DEAE Cellulose	820	123	1.7	11.4	1408	38	21.6
II	Hydroxylapatite	100	22.4	13.7	60.9	1365	37	115
III	Sepharose 6B-CL	15	2.9	59.8	309	897	24	583

*Units = pmol T3 min^{-1}

24% (See Table 4). The stability of the purified enzyme depended on 3 major factors: pH, temperature, and the presence of sulfhydryl reducing equivalents. The purified enzyme rapidly precipitated at pH's below 5.8 and the 5'-deiodinase activity was not stable above pH 8.0. The 5'-deiodinase was more stable at 4 °C than at room temperature, however, the purified 5'-deiodinase could not be frozen without the formation of an insoluble protein precipitate. The addition of dithiothreitol or mercaptoethanol had a stabilizing effect on the activity. When Fraction III (pH 6.5) was stored in capped vials at 4 °C in the presence of 10 mmol/L mercaptoethanol, up to 35 % of the 5'-deiodinase activity was lost over a 2 week period.

## Characterization of Purified Iodothyronine 5'-Deiodinase

Unless otherwise indicated, the following characterization studies were performed on the purified Fraction III preparations within 2 weeks of their purification. Different enzyme preparations were purified from 450- to 600-fold relative to the crude rat liver homogenate.

Determination of Molecular Weight

When the molecular weight of the iodothyronine 5'-deiodinase was approximated by analytical gel filtration chromatography using a Sephacryl S-300 superfine column, a single sharp peak of activity eluted from the column between the elution volumes of catalase (232,000 daltons) and ferritin (440,000 daltons), and a molecular weight of 250,000 was estimated for the detergent solubilized deiodinase complex on the basis of its elution volume relative the protein standards (Figure 12). The

Figure 12. Estimation of apparent molecular weight for iodothyronine 5'-deiodinase by gel filtration chromatography on Sephacryl S-300 sf in the presence of 0.1 % Nonidet P-40.

Details of the experimental procedure are described in Materials and Methods. The marker proteins used were: ribonuclease A (13,700), chymotrypsinogen A (25,000), ovalbumin (43,000), bovine serum albumin (67,000), aldolase (158,000), catalase (232,000), ferritin (440,000) and thyroglobulin (669,000). The apparent molecular weight of the NP-40 solubilized iodothyronine 5'-deiodinase complex was estimated to be approximately 250,000.
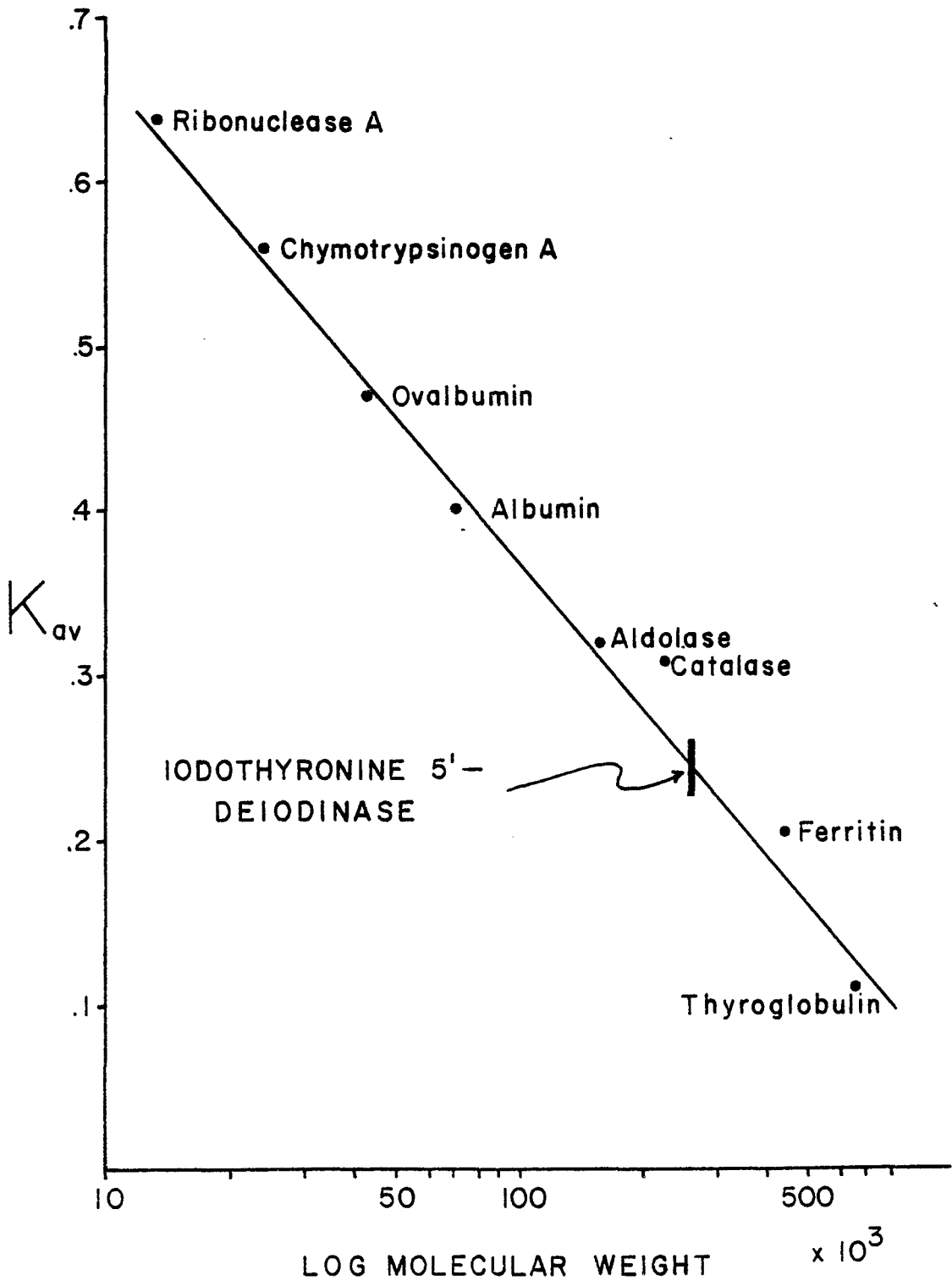
Stokes radius of the enzyme was estimated to be 5.8 nm. The molecular weight of the enzyme was also estimated to be approximately 250,000 when chromatography was performed using Sephadex G-200 and approximately 280,000 when chromatography was performed using Sepharose 6B-CL.

Isoelectric Focusing of the Nonidet P-40 Solubilized 5'-Deiodinase

The isoelectric point (pI) of the Fraction III iodothyronine 5'-deiodinase was determined by column electrofocusing in a sucrose stabilized gradient of pH 3-10. The major activity peak (Figure 13) had an apparent pI of 7.4. Minor peaks of activity were observed in the acidic and basic ends of the pH gradient, however, these were considered to be artifactual. When the column polarity was reversed and the vertical column was run in a descending rather than an ascending manner, a single major activity peak with a pI of 7.4 was observed and the activity in the acidic range completely disappeared. As a result of denaturation during electrofocusing, recoveries of the Fraction III deiodinase were from 3 to 10 %.

Effect of pH and Temperature

The conversion of T4 to T3 by the detergent solubilized 5'-deiodinase was dependent on pH and temperature. The optimal pH was found to pH 6.5 (Figure 14,A) and the optimal temperature was determined to be 37 °C (Figure 14,B). There was a sharp drop in activity below pH 5.8, and the enzyme demonstrated decreased stability above 37 °C. At 56 °C, the enzyme lost 50 % of its activity within 3 minutes (Figure 15). A 30 minute preincubation at 56 °C completely inactivated the 5'-deiodinase.

Figure 13.   Isoelectric focusing of iodothyronine 5'-deiodinase in the presence of 0.1 % Nonidet P-40.

Isoelectric focusing of a 3.0 mg pool of Fraction III protein on a sucrose gradient was performed in a LKB 8100 electrofocusing column. The light gradient solution contained the sample protein, 1.25 mL of ampho-lytes (pH 3.5-10), 0.1 % NP-40, and 5 mmol/L dithiothreitol in a total volume of 60 mL. The heavy gradient solution contained 46.6 % sucrose, 3.75 mL ampholytes (pH 3.5-10), 0.1 % NP-40, and 5mmol/L dithiothreitol in a final volume of 60 mL. After forming the sucrose gradient, the sam-ple was focused 48 hours (4 °C) at a final 400 volts, keeping power less than 1.5 watts throughout, and was eluted with the assistance of a peri-staltic pump into 1.5 mL fractions. $\triangle$——$\triangle$, 5'-deiodinase activity; ●———● , protein concentration (Bradford); ······· , pH. The resolved peak of iodothyronine 5'-deiodinase activity had a pI of 7.4.
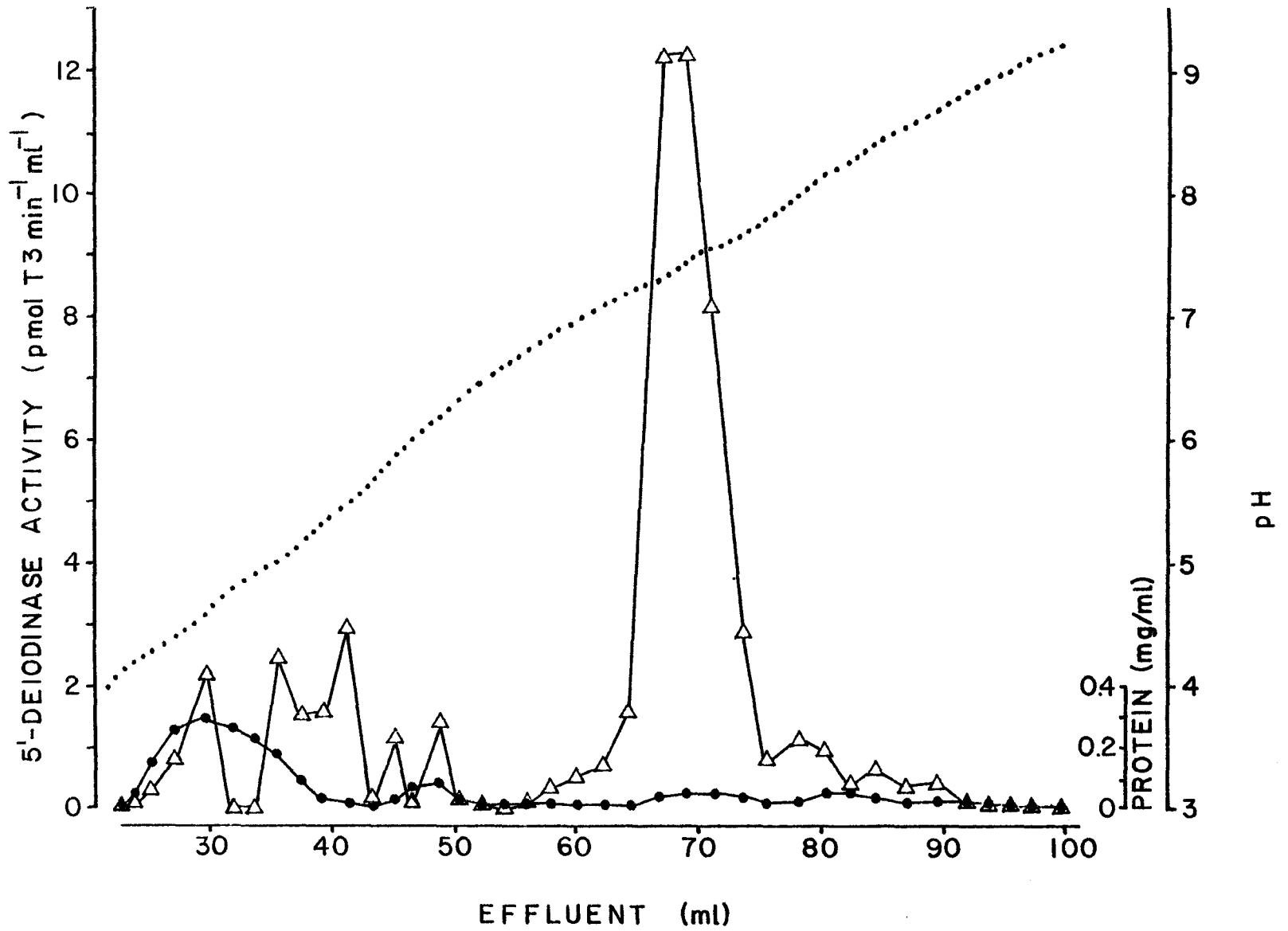
Figure 14.   Effect of pH and temperature on iodothyronine 5'-deiodinase activity.

Enzyme assays were varied from standard reaction conditions as indicated. The rate of T3 formation from the deiodination of T4 by Fraction III enzyme was plotted as the mean ± standard deviation for triplicate determinations. A. Enzyme assays were buffered with 0.15 mol/L citrate phosphate (pH 4.0-6.0), 0.15 mol/L potassium phosphate (pH 6.0-7.5) or 0.15 mol/L Tris-HCL (pH 7.5-9.0). In separate experiments, the citrate and potassium phosphate buffers gave similar results at pH 6.0, and the phosphate and Tris buffers gave similar results at pH 7.5. B. Enzyme assays were carried out in water baths thermostated to: 25, 37, 41, 45, 51, and 56 °C.

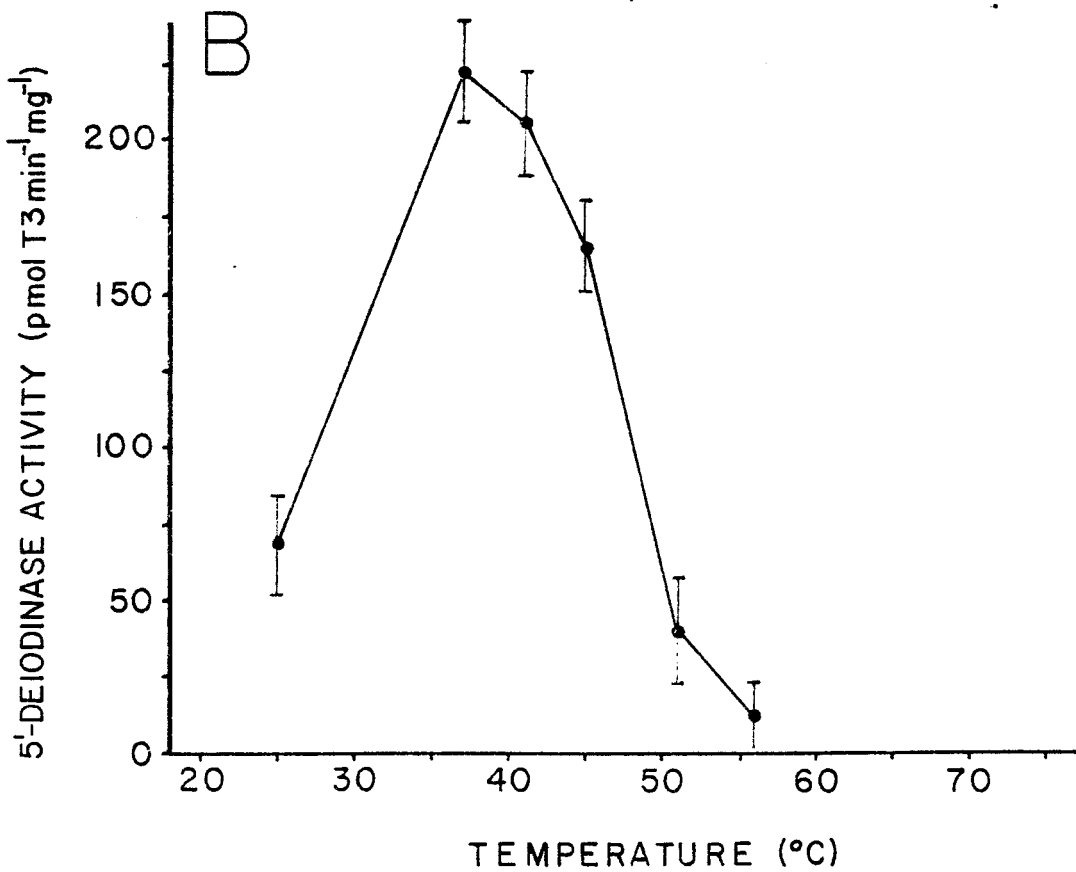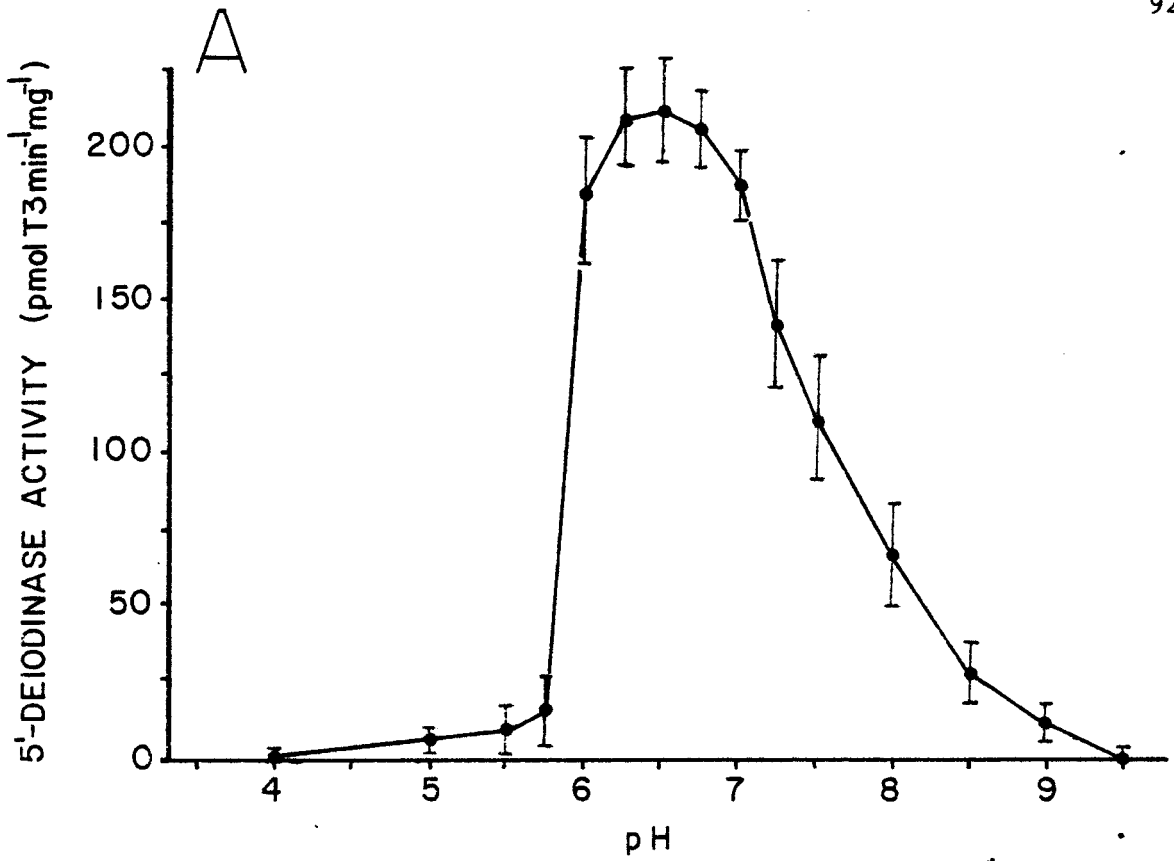Figure 15. Thermal inactivation of iodothyronine 5'-deiodinase at 56 °C.

Preincubation of Fraction III at 56 °C was carried out for time intervals of 0 to 40 min. Following preincubation, aliquots of the enzyme mixture were assayed at 37 °C for 30 min to determine the amount of T4 to T3 converting activity remaining. Data are plotted as the mean ± standard deviation of triplicate determinations.
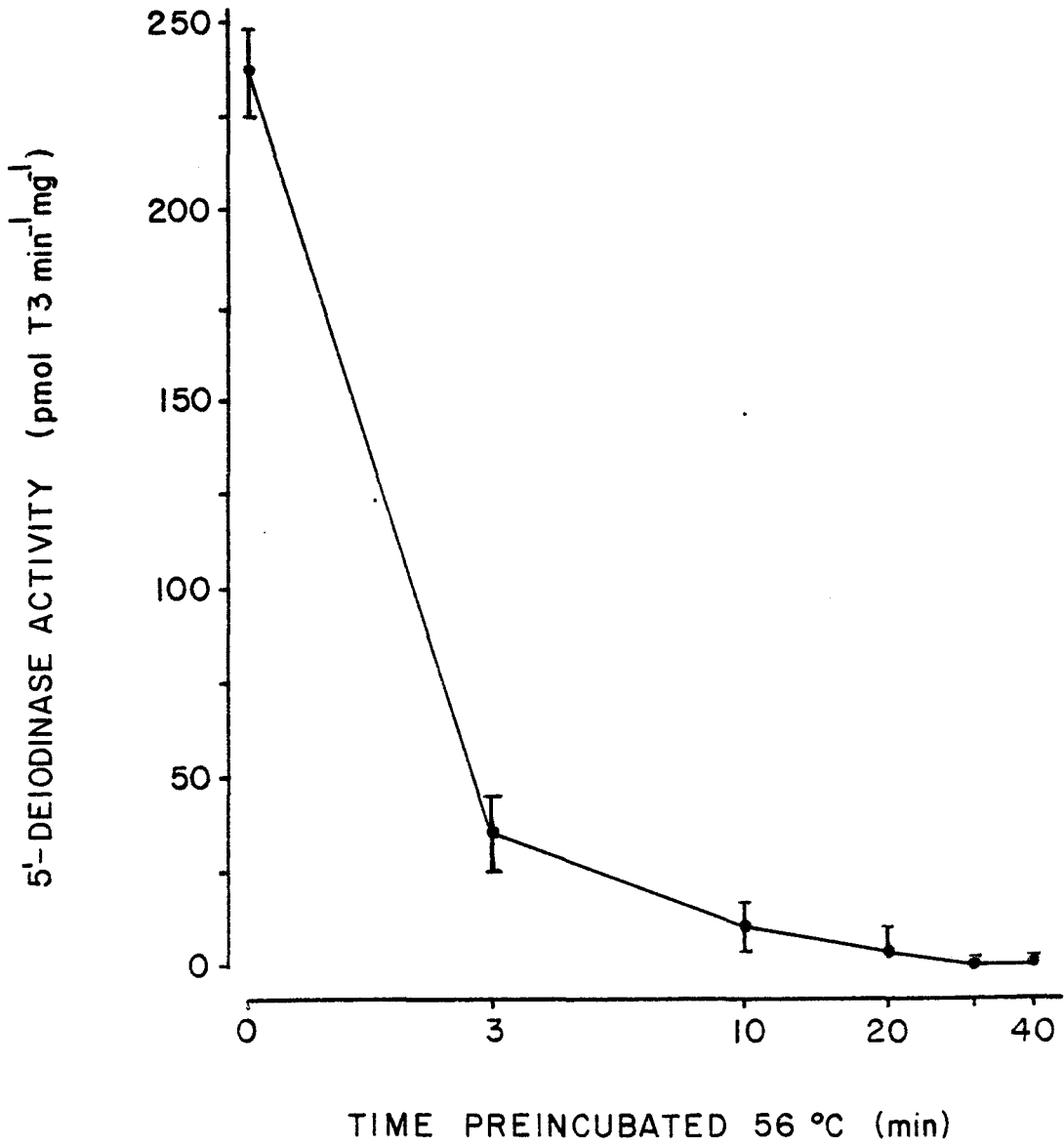
Linearity With Time and Protein Concentration

A progressive increase of T3 formation with time (Figure 16, A) and
with protein concentration (Figure 16, B) was demonstrated for the deter-
gent solubilized 5'-deiodinase. The increase with time appeared linear
for the first 60 minutes and gradually fell off thereafter. Linearity of
activity with protein concentration was observed when velocity determi-
nations were corrected for detergent inhibition. In contrast, activity
of the crude rat liver homogenate Fractions A and D was not linear with
protein concentration. Dilution of these fractions resulted in a 3- to
5-fold increase in specific activity.

Effect of Substrate and Detergent Concentration

Figure 17 shows the interrelationship of thyroxine concentration
and NP-40 concentration on the conversion of T4 to T3 by the partially
purified iodothyronine 5'-deiodinase (Fraction II). In the absence of
detergent, increasing the substrate concentration from 1 μmol/L T4 to 12
μmol/L T4 resulted in the formation of progressively larger amounts of
T3. Further increases above 12 μmol/L T4 resulted in substrate inhibi-
tion. The inhibitory effect of high concentrations of T4 was decreased
when detergent was present in the assay mixture although increasing the
concentration of detergent also had an inhibitory effect on 5'-deiodinase
activity. The effect of Nonidet P-40 concentration on enzyme activity at
a constant concentration of T4 is shown in Figure 18. Nonidet P-40 at a
concentration of 0.32 % decreased the activity by 50 %.

In order to compare the inhibitory effect of Nonidet P-40 to that
of deoxycholate (DOC), the activity of Fraction II in the presence of DOC

Figure 16. Linearity of iodothyronine 5'-deiodinase activity with time and protein concentration.

  The rate of T3 formation from the deiodination of T4 by Fraction III was plotted as the mean ± standard deviation for triplicate determinations. A. Enzyme assays were incubated at 37 °C for various periods of time from 0 to 4 hours. B. Increase in volumes of Fraction III containing from 2 to 25 μg of protein were added to the enzyme assay mixture -----, actual 5'-deiodinase activity observed; ——— , activity corrected for Nonidet P-40 inhibition.
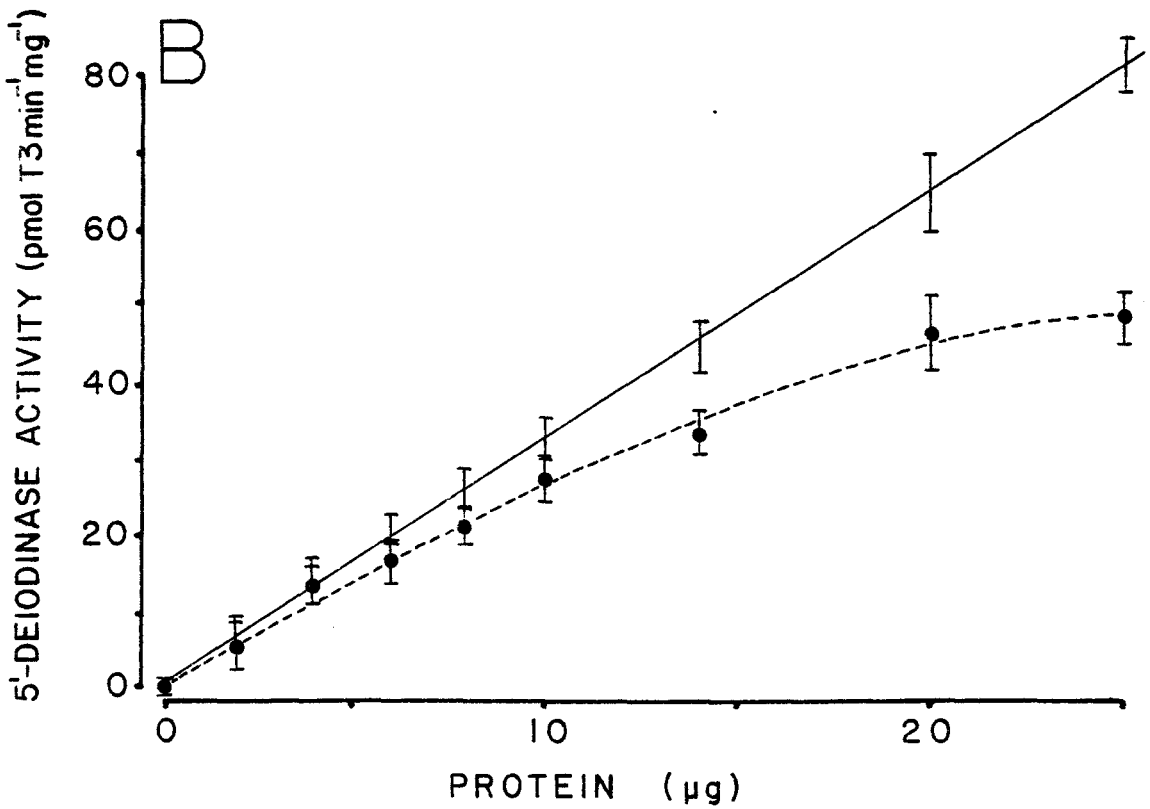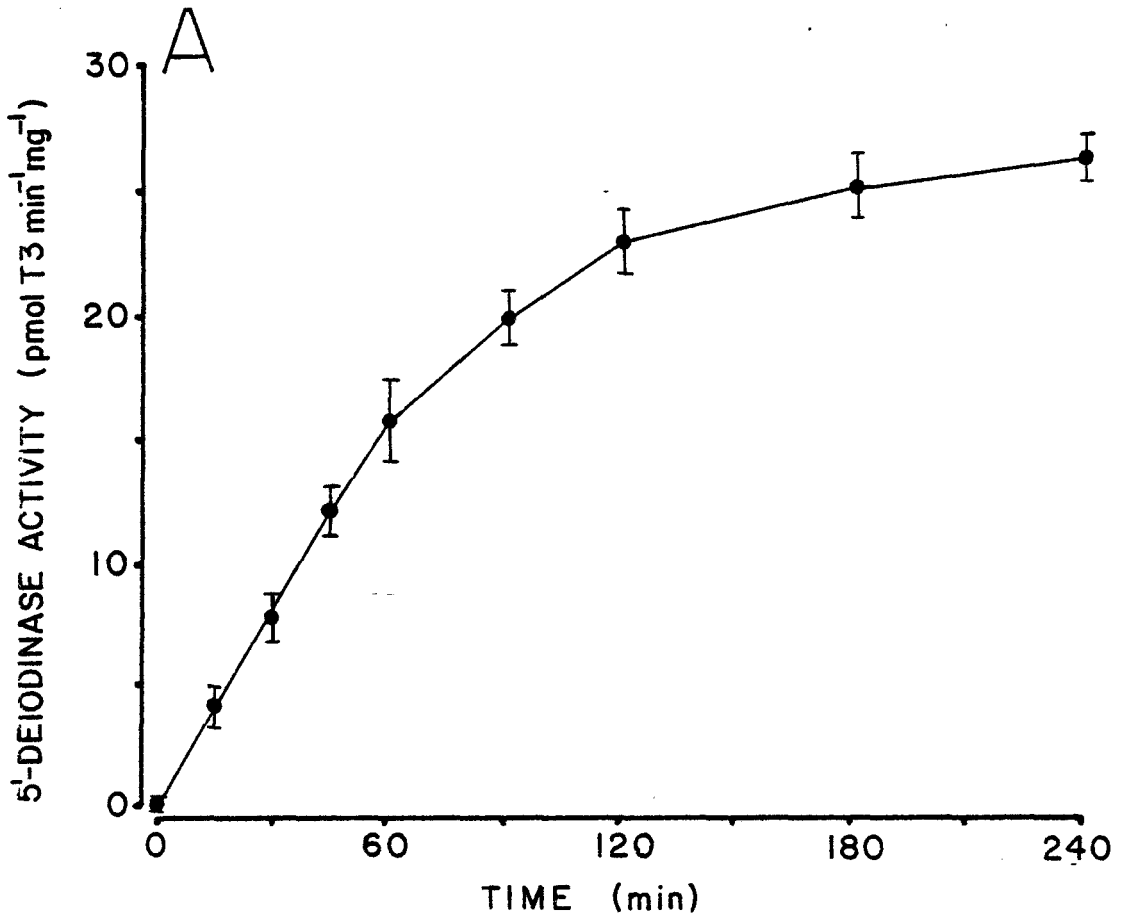
Figure 17. Substrate saturation of iodothyronine 5'-deiodinase activity in the presence and absence of Nonidet P-40.

   100 µL aliquots of Fraction II preparations containing: o——o , 0.0 % Nonidet P-40; ●——● , 0.1 % Nonidet P-40; ▲——▲ , 0.2 % Nonidet P-40; and ■——■ , 0.5 % Nonidet P-40 were added to enzyme assay mixtures of 1.0 mL final volume. The T4 and T3 converting activity, as a function of T4 concentration for each detergent level was plotted as the mean ± standard deviation for triplicate determinations.
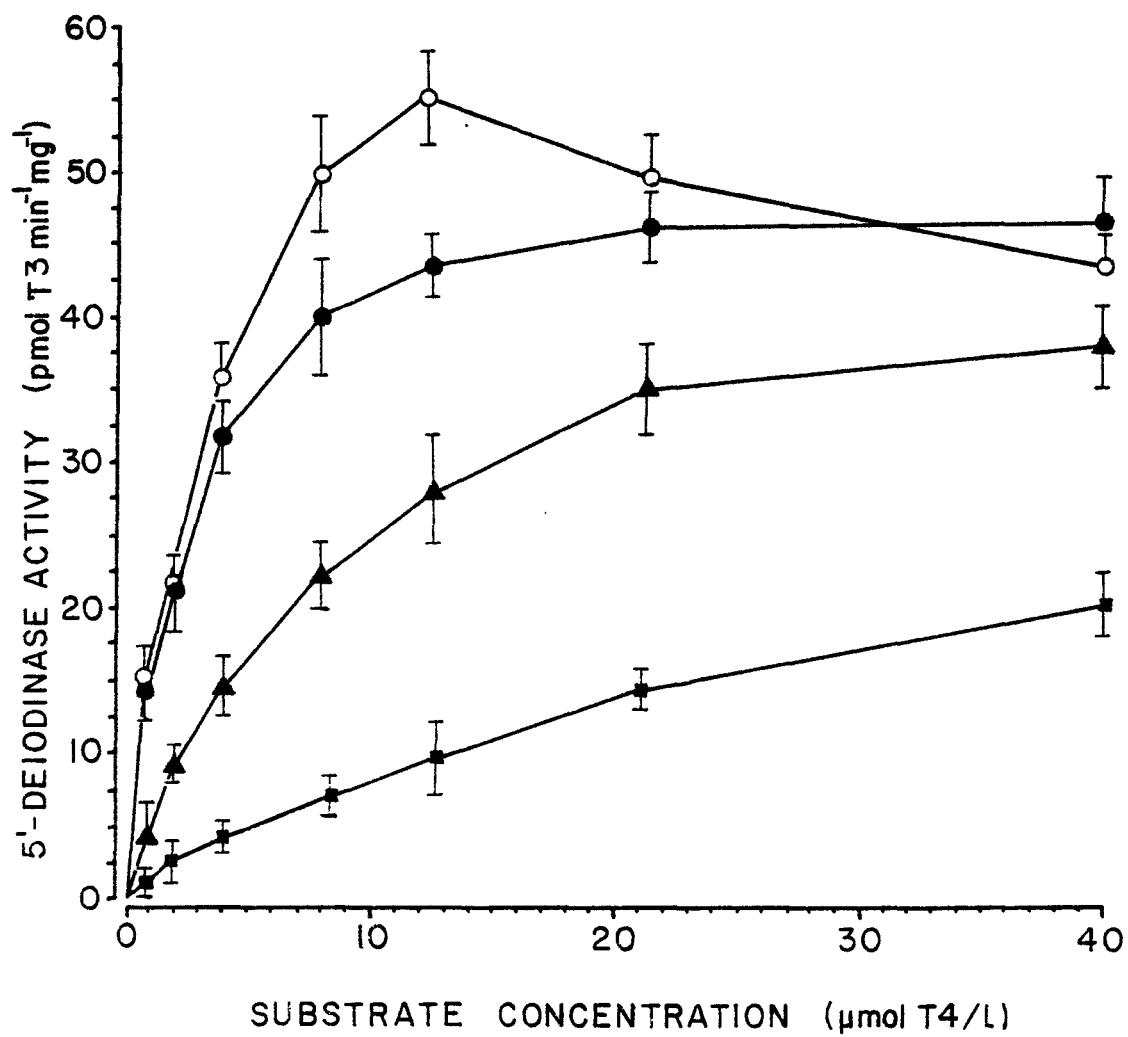
Figure 18. Effect of Nonidet P-40 and deoxycholate on iodothyronine
5'-deiodinase activity.

    100 μL aliquots of Fraction II containing increasing concentrations
of Nonidet P-40 from 0.025 to 1.0 % (w/v) or of deoxycholate from 0.05 to
0.4 % (w/v) were incubated under standard enzyme assay conditions (1.0 mL
final volume). Iodothyronine 5'-deiodinase activity was plotted as the
mean ± standard deviation for triplicate determinations. ●——● , %
activity in the presence of Nonidet P-40; o——o , % activity in the
presence of deoxycholate.
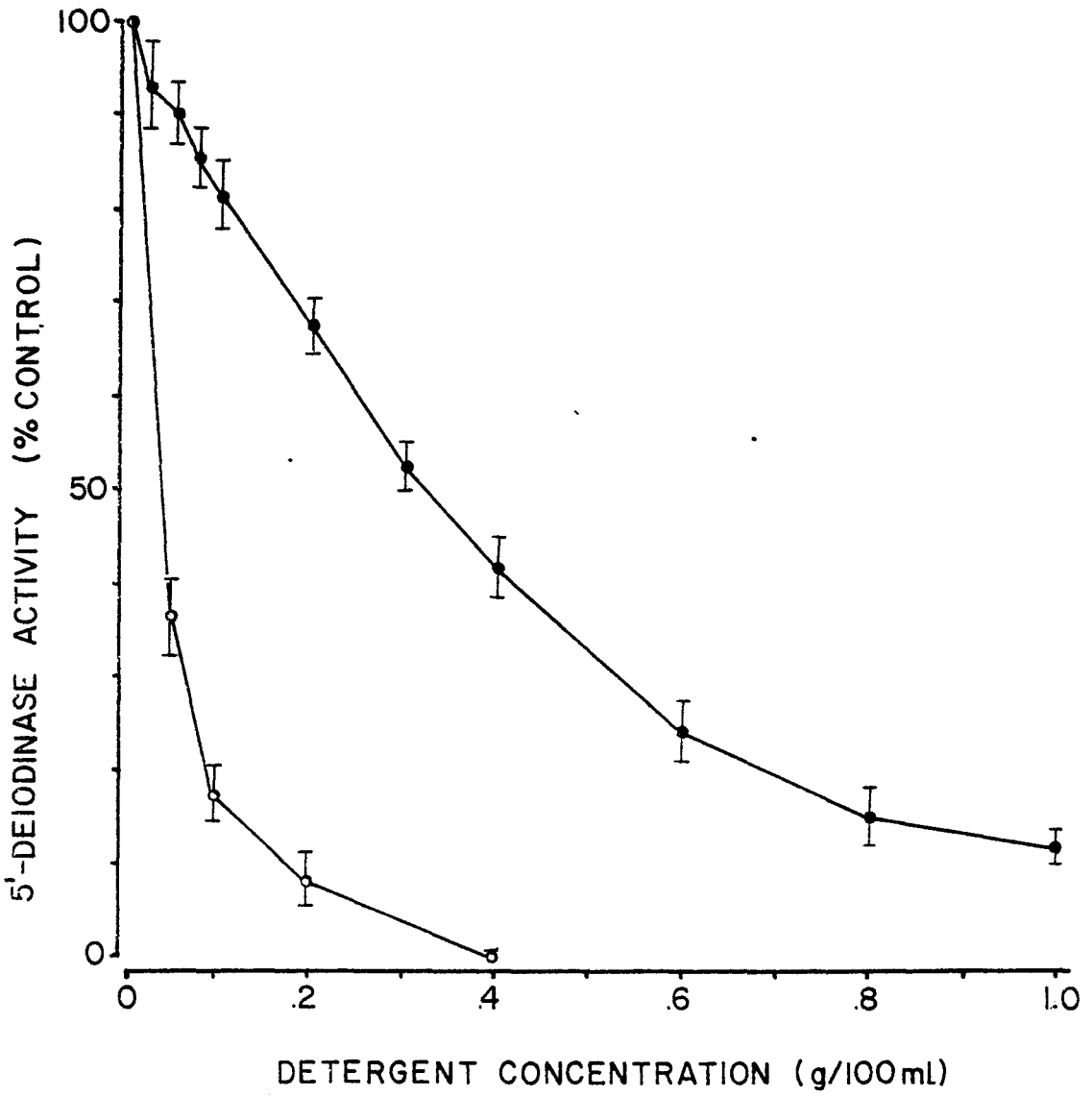
was also studied. When DOC was added to Fraction II at concentrations
of: 0.05, 0.1, 0.2, 0.4, and 0.8 %, the 5'-deiodinase activity was de-
creased by 64, 83, 92, and 100 %, respectively (See Figure 18).

Activators and Inhibitors

Several thiol group-containing compounds were found to activate the
5'-deiodinase in a concentration dependent manner and to be essential for
activity (Figure 19). Dithiothreitol had the greatest stimulatory ef-
fect, which plateaued at a concentration of around 20 mmol/L. Beta-
mercaptoethanol, reduced glutathione, cysteine, and lipoamide also had a
stimulatory effect on the T4 to T3 converting activity. ·

A number of different agents were tested for possible effects on
the 5'-deiodinase activity of Fraction III (Table 5). The deiodinase had
no apparent requirement for the presence of metal cofactors or the pre-
sence of other cations or anions. EDTA at a 1.0 mmol/L concentration did
not significantly effect deiodinase activity. Several salts including:
potassium chloride, lithium chloride, sodium iodide, magnesium sulfate,
zinc acetate, mercury chloride and lead nitrate had an inhibitory effect
on deiodinase activity. Of these compounds, zinc acetate was the most
potent inhibitor. Mercury chloride and lead nitrate were not potent in-
hibitors of the 5'-deiodinase in this study due to the presence of 20
mmol/L dithiothreitol, however, when crude rat liver homogenates were
assayed in the absence of dithiothreitol, the addition of 0.2 mmol/L lead
nitrate to the enzyme assay mixture completely inhibited the deiodinase
activity. This inhibition could be overcome in a competitive manner with
the addition of dithiothreitol. The conversion of T4 to T3 was also

Figure 19. Effect of various sulfhydryl reagents on iodonthronine 5'-deiodinase activity.

Fraction III was used as the source of the 5'-deiodinase activity. Assays were done under standard conditions (19.3 μmol/L T4) except for the variation in cofactor and cofactor concentration. o——o , dithiothreitol (DTT); ●——● , Lipoamide; △——△ , beta mercaptoethanol (BME); ▲——▲ , reduced glutathione (GSH); □——□ , cysteine (Cys).
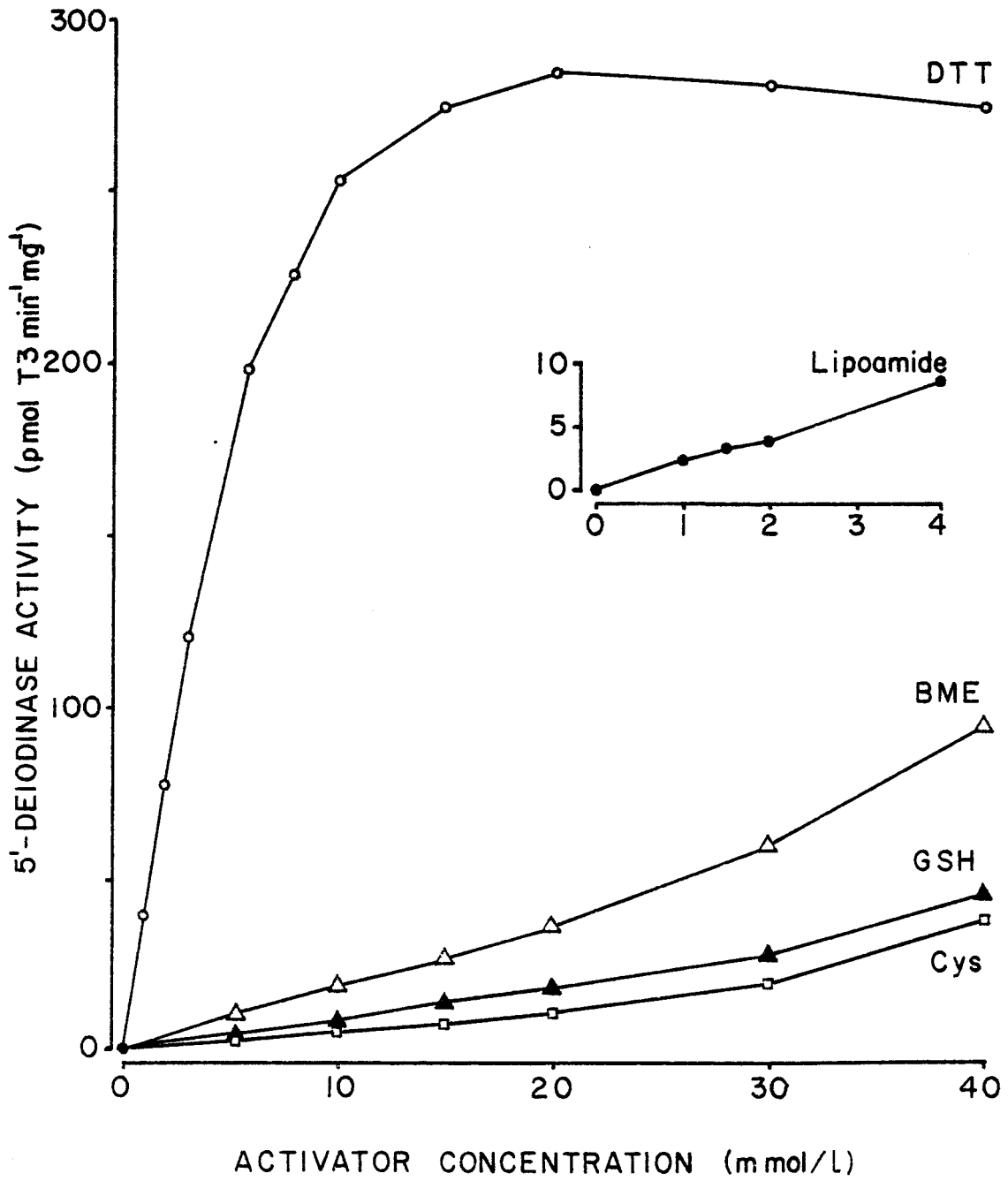
Table 5

Effect of Various Agents on Fraction III

5'-Deiodinase Activity

TEST AGENT	CONCENTRATION	% OF CONTROL* ACTIVITY
EDTA	1.0 mmol/L	96 ± 8
NaCL	100.0 mmol/L	104 ± 9
KCL	10.0 mmol/L 20.0 mmol/L	96 ± 5 82 ± 7
LiCL	1.0 mmol/L 5.0 mmol/L	92 ± 6 78 ± 5
$MgCL_2$	5.0 mmol/L	106 ± 9
$CaCL_2$	1.0 mmol/L	102 ± 10
$MnCL_2$	1.0 mmol/L	96 ± 9
$Zn(C_2H_3O_2)_2$	0.1 mmol/L 1.0 mmol/L	21 ± 4 4 ± 2
$HgCL_2$	0.1 mmol/L	96 ± 9
$Pb(NO_3)_2$	0.1 mmol/L	85 ± 7
$FeCL_3$	0.8 mmol/L	98 ± 9
NaI	5.0 mmol/L 10.0 mmol/L	94 ± 8 82 ± 7
$MgSO_4$	1.0 mmol/L 5.0 mmol/L	88 ± 7 48 ± 8
NaN_3	5.0 mmol/L	99 ± 10
human serum	15 mg protein/ml	8 ± 2
bovine serum albumin	15 mg protein/ml	38 ± 4

* mean ± standard deviation for 5 determinations; standard assay conditions

inhibited by charcoal stripped serum and to a lesser extent, by bovine serum albumin. The activity of the 5'-deiodinase was not significantly activated by any of the compounds tested. It was noted, however, that ferric chloride enhanced the nonenzymatic, spontaneous deiodination of T4 to T3. Sodium azide was found to have no effect on T3 formation.

The T4 to T3 converting activity of Fraction III iodothyronine 5'-deiodinase was unaffected by large doses of dexamethasone and methimazole, however, reverse T3 (rT3), propylthiouracil, propranolol, sodium salicylate, iodipamide (Cholografin), and to a lesser extent, sodium diatrizoate (Hypaque) inhibited 5'-deiodinase activity in a dose-dependent manner (Figure 20). The most potent of these inhibitors was rT3. Kinetic studies indicated that rT3 was an apparent non-competitive inhibitor of the 5'-deiodinase with respect to both T4 and the essential cofactor, dithiothreitol (DTT) (K_I = 0.270 and 0.099 μmol/L, respectively, Figure 21). Propylthiouracil was an apparent uncompetitive inhibitor with respect to T4 and an apparent competitive inhibitor with respect to DTT (K_I = 2.62 and 1.16 μmol/L, respectively, Figure 22). Sodium salicylate, Cholografin, and propranolol were apparent competitive inhibitors of the 5'-deiodinase with respect to both T4 (K_I = 75 μmol/L, 19.2 μmol/L and 0.98 mmol/L, respectively) and DTT (K_I = 143 μmol/L, 10.4 μmol/L, and 2.20 mmol/L, respectively, Figures 23-25).

Determination of Kinetic Parameters and Possible Mechanism of Action

Double reciprocal plots of the Lineweaver-Burke type derived from kinetic data for the Fraction III 5'-deiodinase generated parallel lines when velocity versus T4 substrate concentration was plotted for 4

Figure 20. Comparison of the effect of various inhibitors and related compounds on iodothyronine 5'-deiodinase activity.

Fraction III was incubated under standard enzyme assay conditions with the test agents as indicated. ●———●, reverse T3; o———o, propylthioutacil (PTU); ■———■, iodipamide (cholografin); □———□, sodium salicylate; △———△, propranolol; ▲———▲, sodium diatrizoate (hypaque); ---------, dexamethasone; ...., methimazole (Tapazole). An arbitrary value of 100 was assigned to the amount of T3 produced in the control tubes containing no inhibitor, and that produced in the presence of test agent was expressed as a percentage of control values. Each point is an average of duplicate determinations.
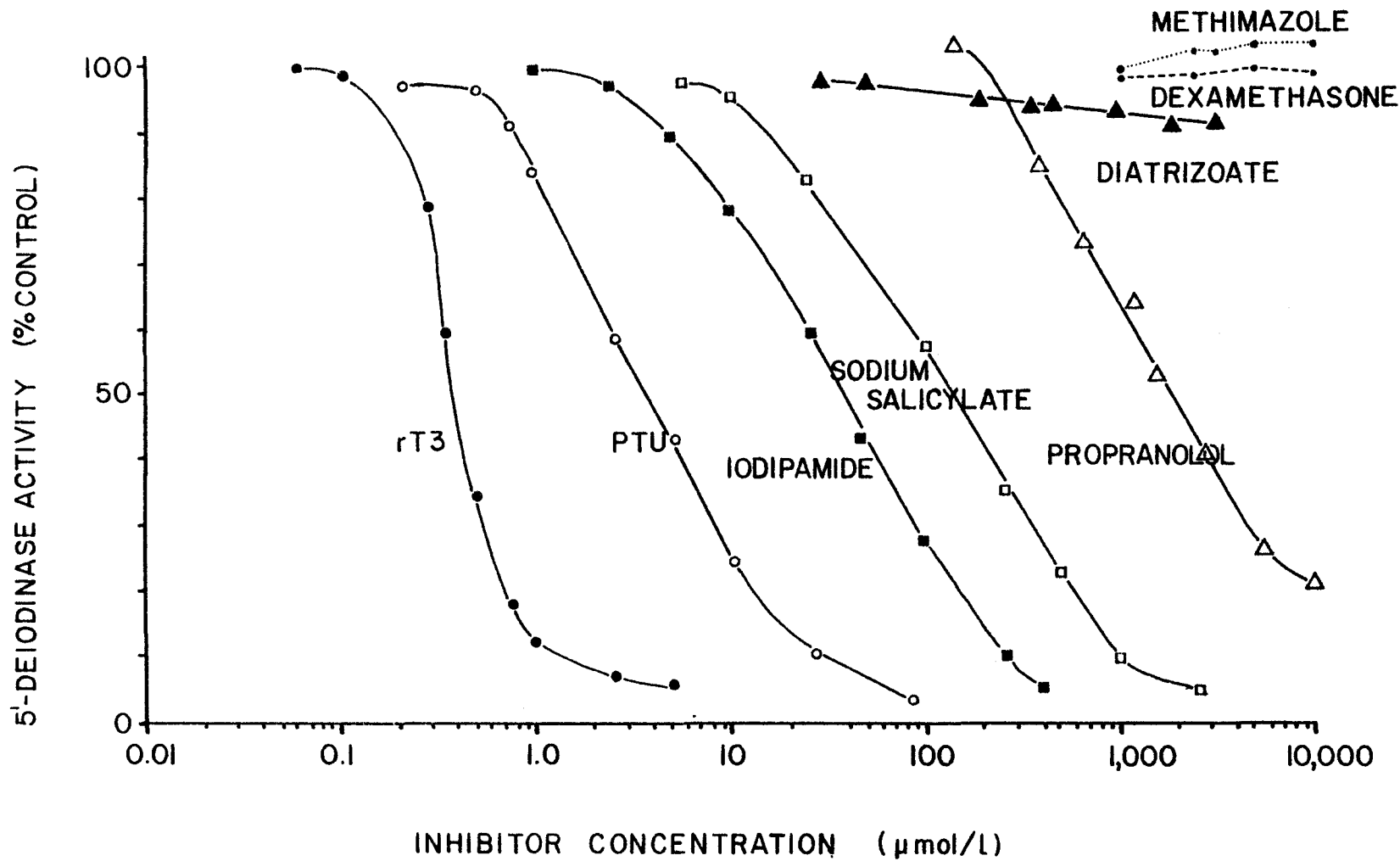
Figure 21. Apparent non-competitive inhibition by rT3 with respect to T4 and apparent non-competitive inhibition by rT3 with respect to dithiothreitol of T4 to T3 conversion by the Fraction III 5'-deiodinase.

Varying quantities of T4 at a constant 20 mmol/L dithiothreitol (A), or varying quantities of dithiothreitol at a constant 19.3 µmol/L T4 (B) were incubated with 100 µL Fraction III (0.01 mg protein) in 0.15 mol phosphate buffer (pH 6.5) for 30 minutes at 37 °C in the absence or presence of rT3. The amount of T3 generated was quantitated by radio-immunoassay of an ethanol extract of the incubation mixture. The T4 to T3 converting activity data was plotted in the form of a Lineweaver-Burke plot: ■——■, 0.0 rT3; ●——●, 0.077 µmol/L rT3; -——- , 0.154 µmol/L rT3; ✗——✗, 0.230 µmol/L rT3; ○——○ , 0.307 µmol/L rT3. Each point represents the average of duplicate determinations. The K_i for rT3 was calculated to be 0.270 µmol/L with respect to T4 and 0.099 µM with respect to dithiothreitol.
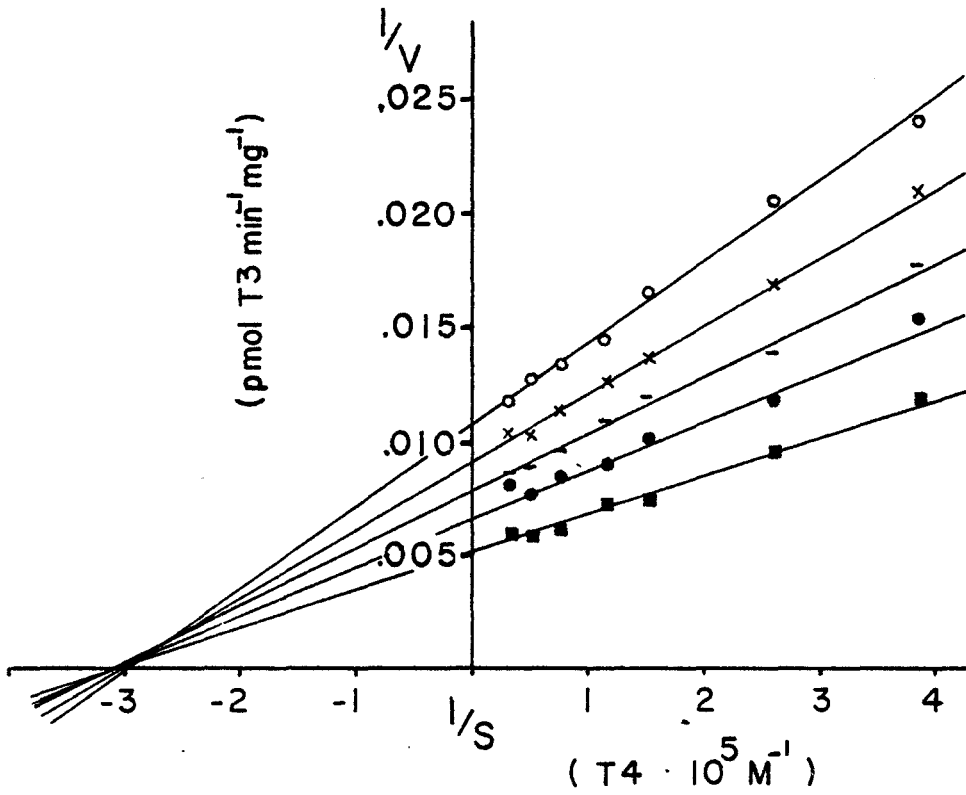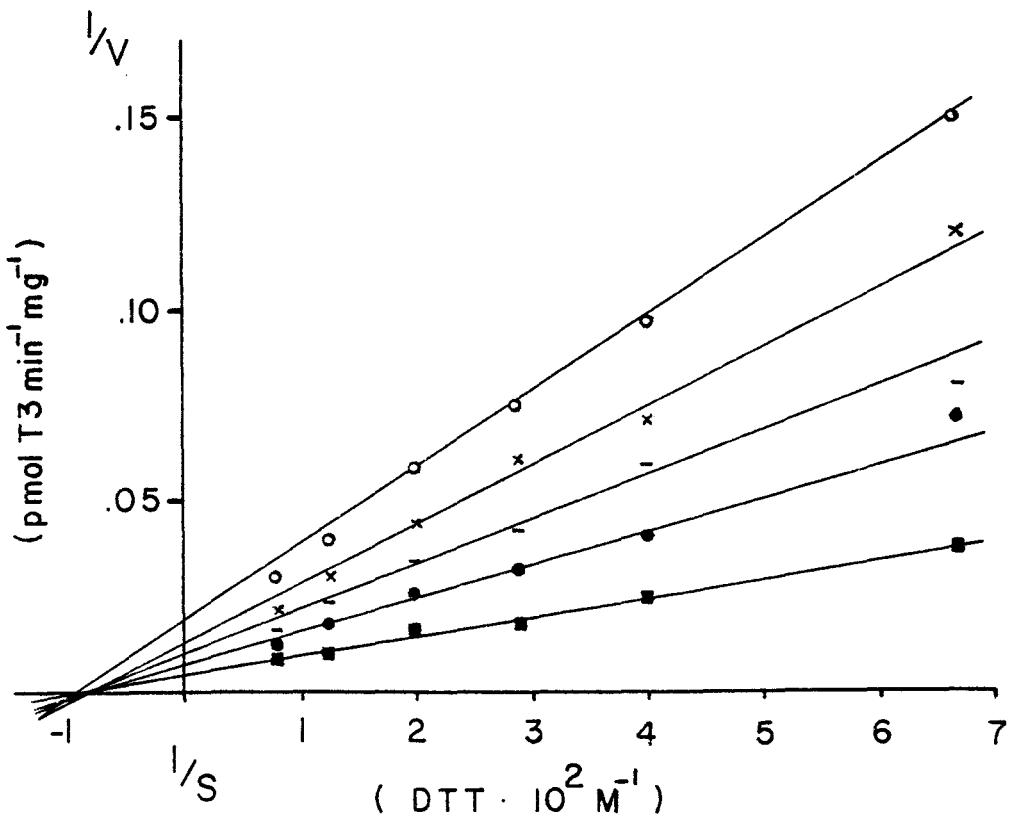
Figure 22. Apparent uncompetitive inhibition by propylthiouracil with respect to T4 and apparent competitive inhibition by propylthiouracil with respect to dithiothreitol of T4 to T3 conversion by the Fraction III 5'-deiodinase.

The experimental detail as well as the manner of plotting the data are described in Figure 21 for: ■———■, 0.0 propylthiouracil (PTU); ●———● , 1.2 μmol/L PTU; -———- , 2.4 μmol/L PTU; ✗———✗ , 3.5 μmol/L PTU; ○———○ , 4.7 μmol/L PTU. The K_i for PTU was calculated to be 2.62 μmol/L with respect to T4 and 1.16 μmol/L with respect to dithiothreitol.
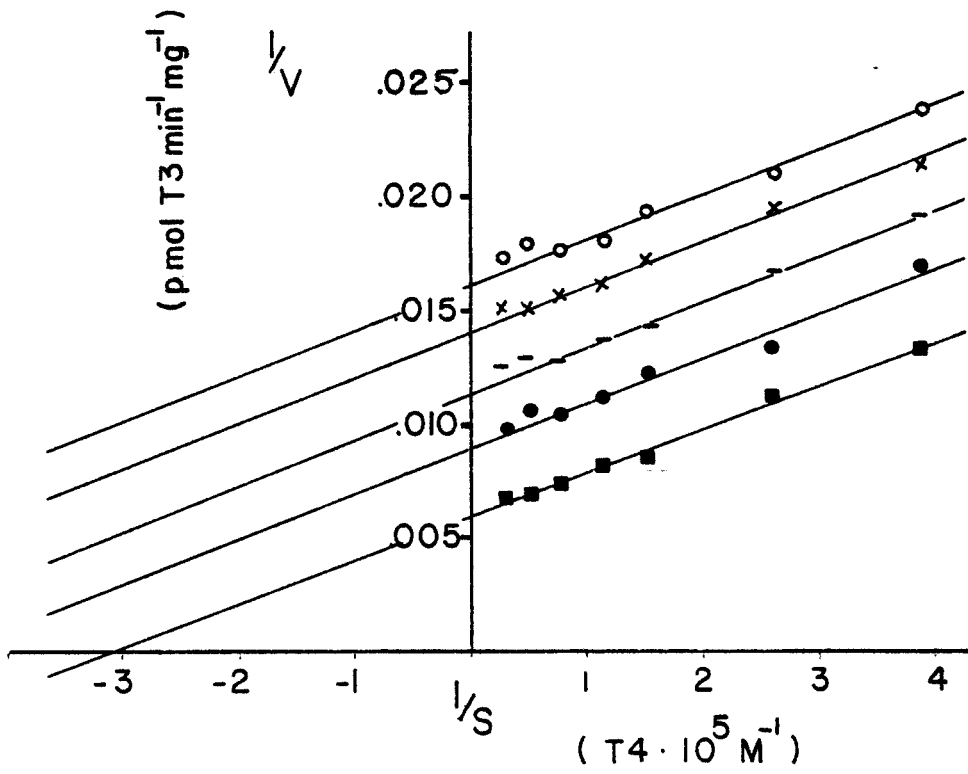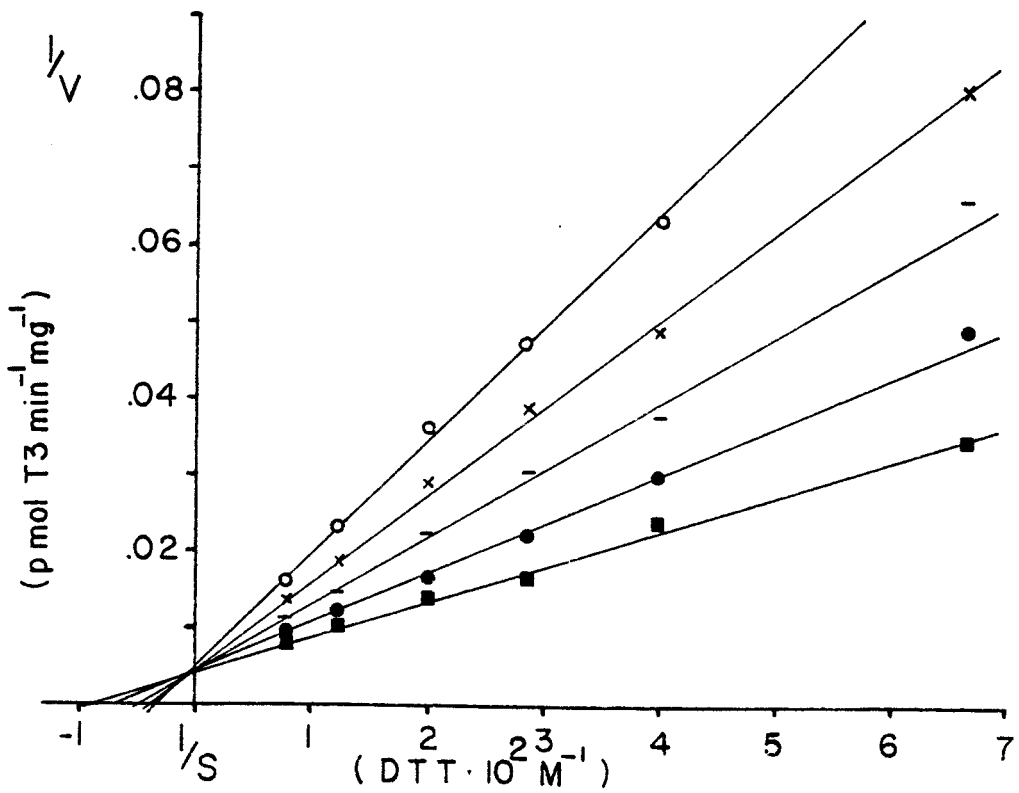
Figure 23. Apparent competitive inhibition by iodipamide (Cholografin) with respect to both T4 and dithiothreitol of T4 to T3 conversion by the Fraction III 5'-deiodinase.

The experimental detail as well as the manner of plotting are described in Figure 21 for: ■——■, 0.0 iodipamide; ●——●, 4.5 μmol/L iodipamide; -——-, 9.0 μmol/L iodipamide; ✗——✗, 13.6 μmol/L iodipamide; ○——○, 18.1 μmol/L iodipamide. The K_i for iodipamide was calculated to be 19.2 μmol/L with respect to T4, and 10.4 μmol/L with respect to dithiothreitol.
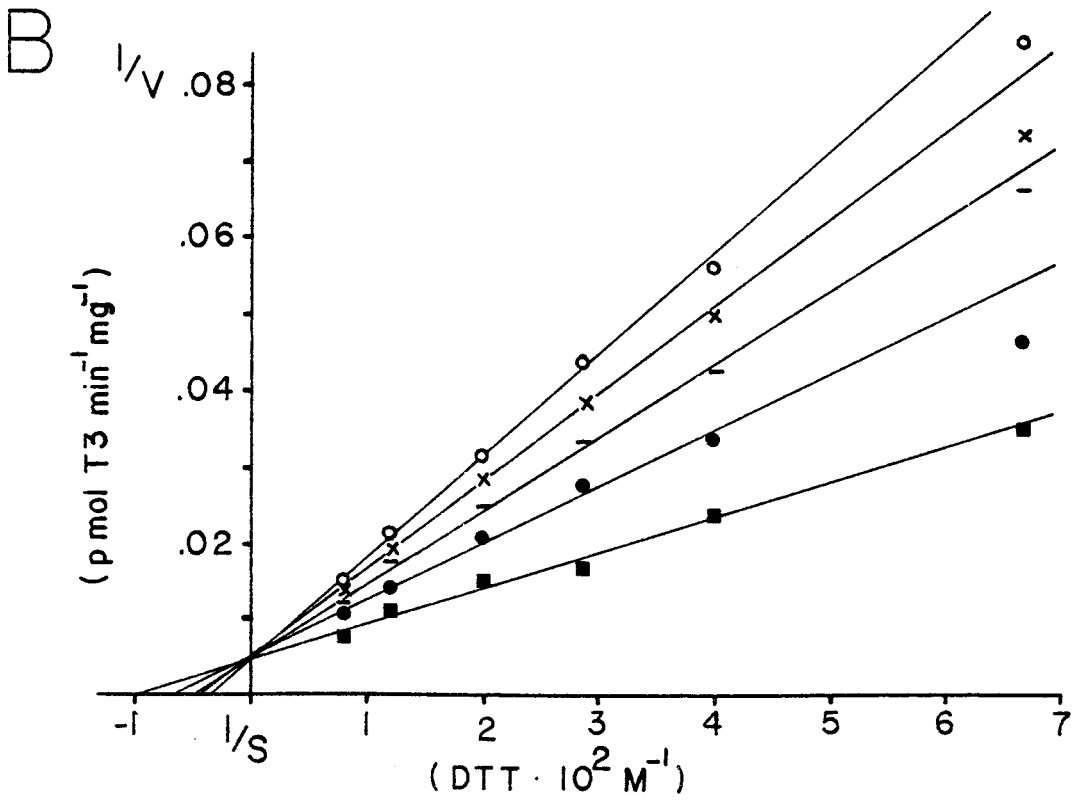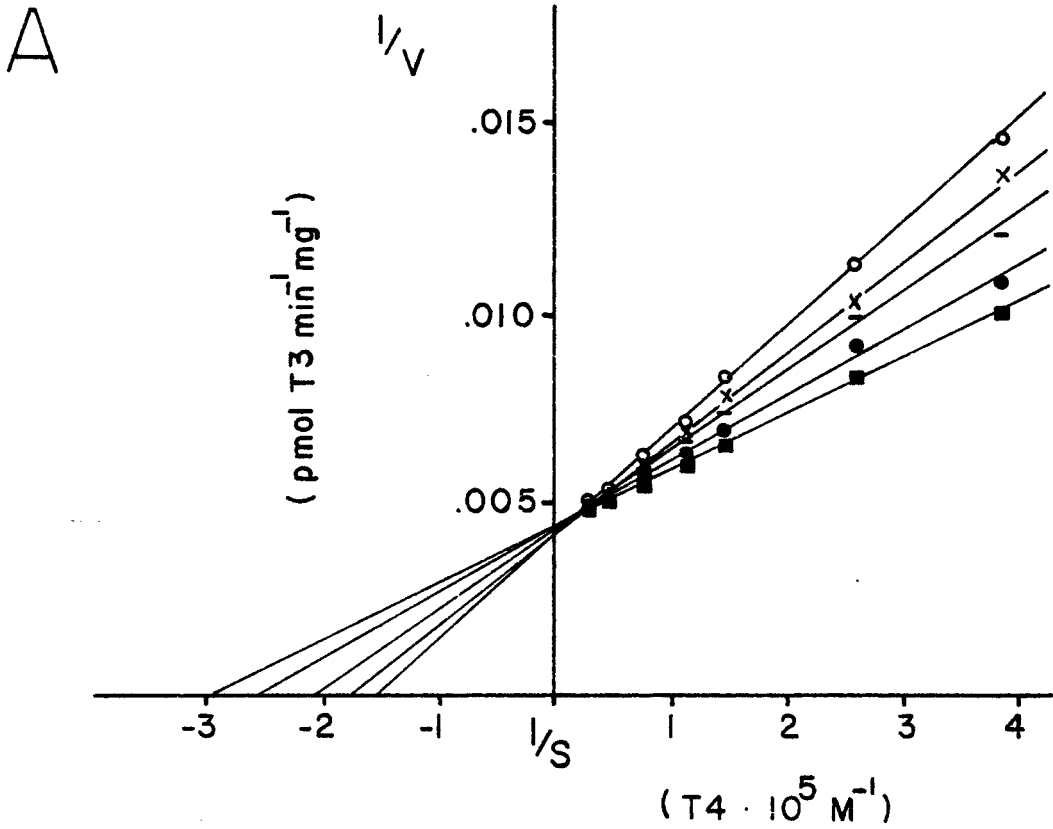
Figure 24. Apparent competitive inhibition by sodium salicylate with respect to both T4 and dithiothreitol of T4 to T3 conversion by the Fraction III 5'-deiodinase.

The experimental detail as well as the manner of plotting are described in Figure 21 for: ■———■ , 0.0 sodium salicylate; ●———● , 125 μmol/L sodium salicylate; –———– , 250 μmol/L sodium salicylate; ✗———✗ , 375 μmol/L sodium salicylate; ○———○ , 500 μmol/L sodium salicylate. The K_i for sodium salicylate was calculated to be 75 μmol/L with respect to T4, and 143 μmol/L with respect to dithiothreitol.
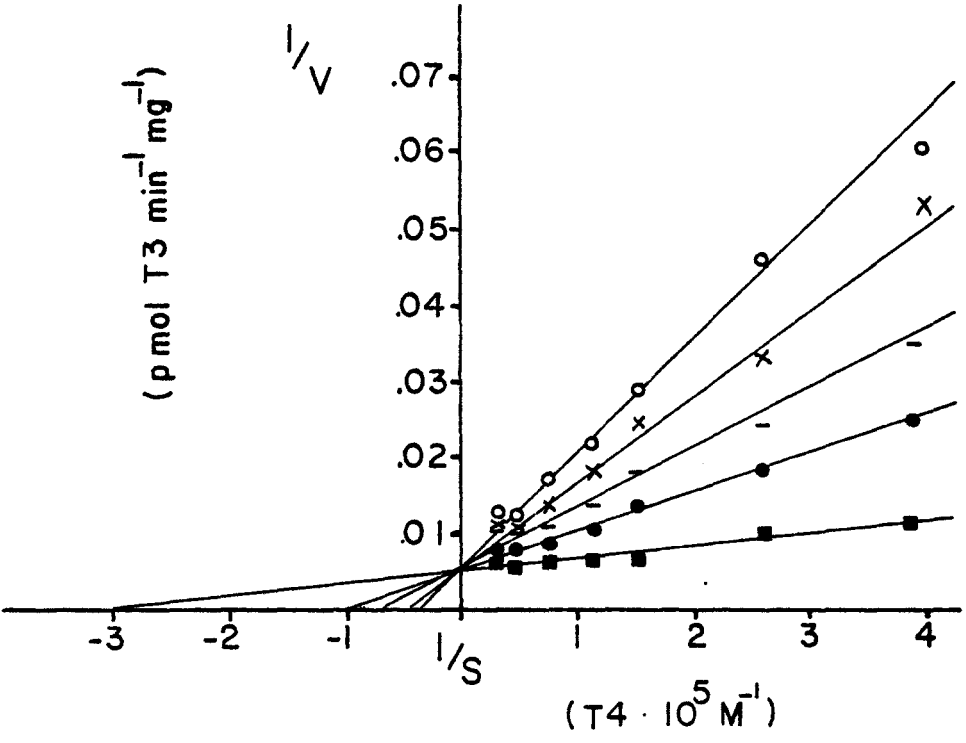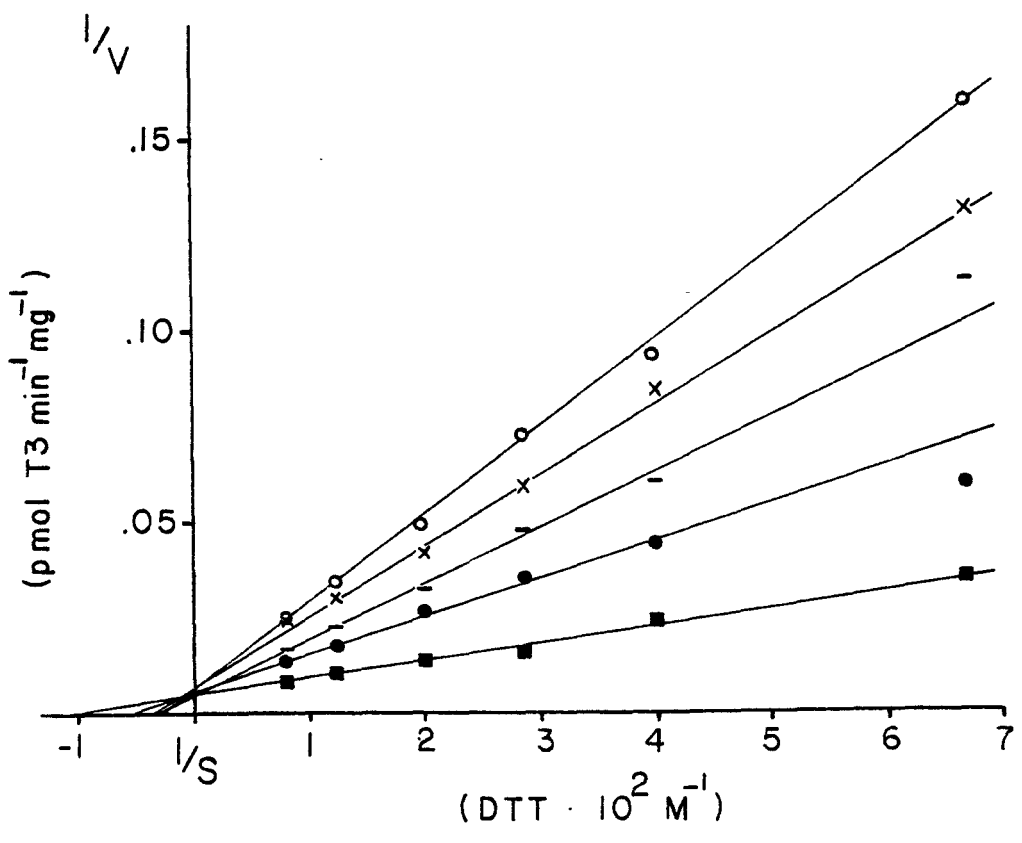
Figure 25. Apparent competitive inhibition by propranolol with respect to both T4 and dithiothreitol of T4 to T3 conversion by the Fraction III 5'-deiodinase.

The experimental detail as well as the manner of plotting are described in Figure 21 for: ■———■ , 0 propranolol; ●———● , .0.42 mmol/L propranolol; – ——–ˑ, 0.84 mmol/L propranolol; ✗———✗ , 1.27 mmol/L propranolol; ○———○ , 1.69 mmol/L propranolol. The K_i for propranolol was calculated to be 0.98 mmol/L with respect to T4, and 2.20 mmol/L with respect to dithiothreitol.
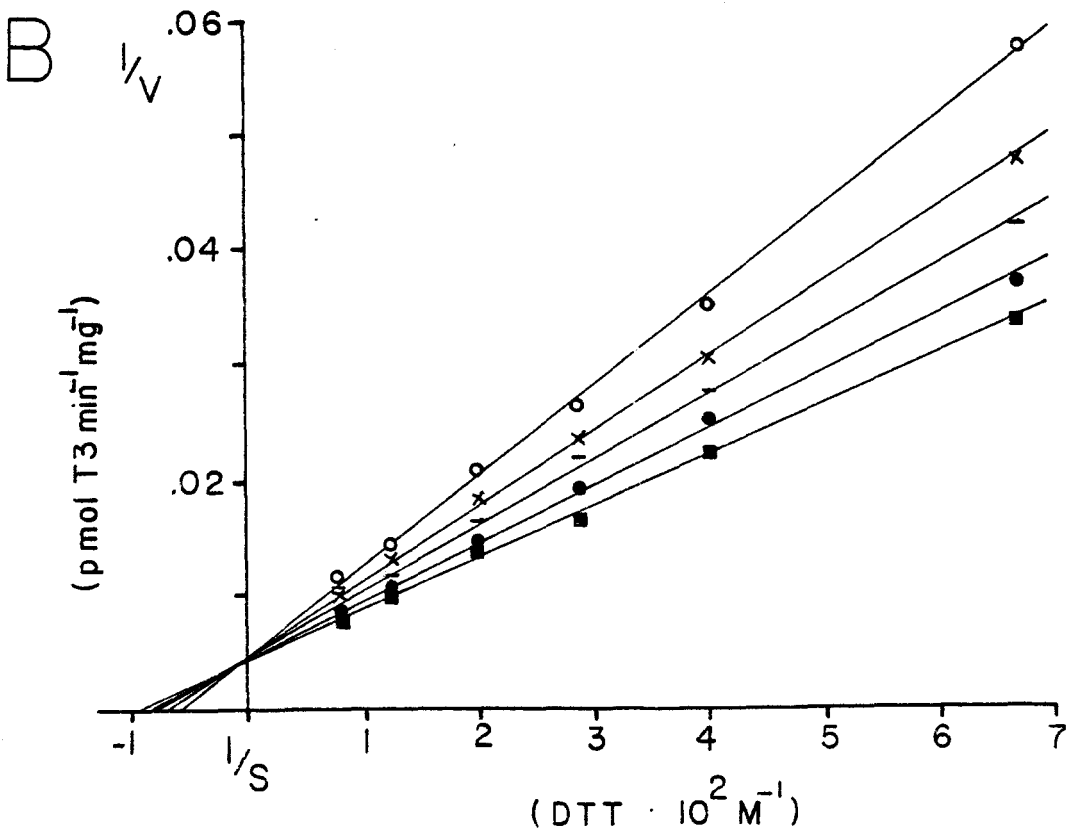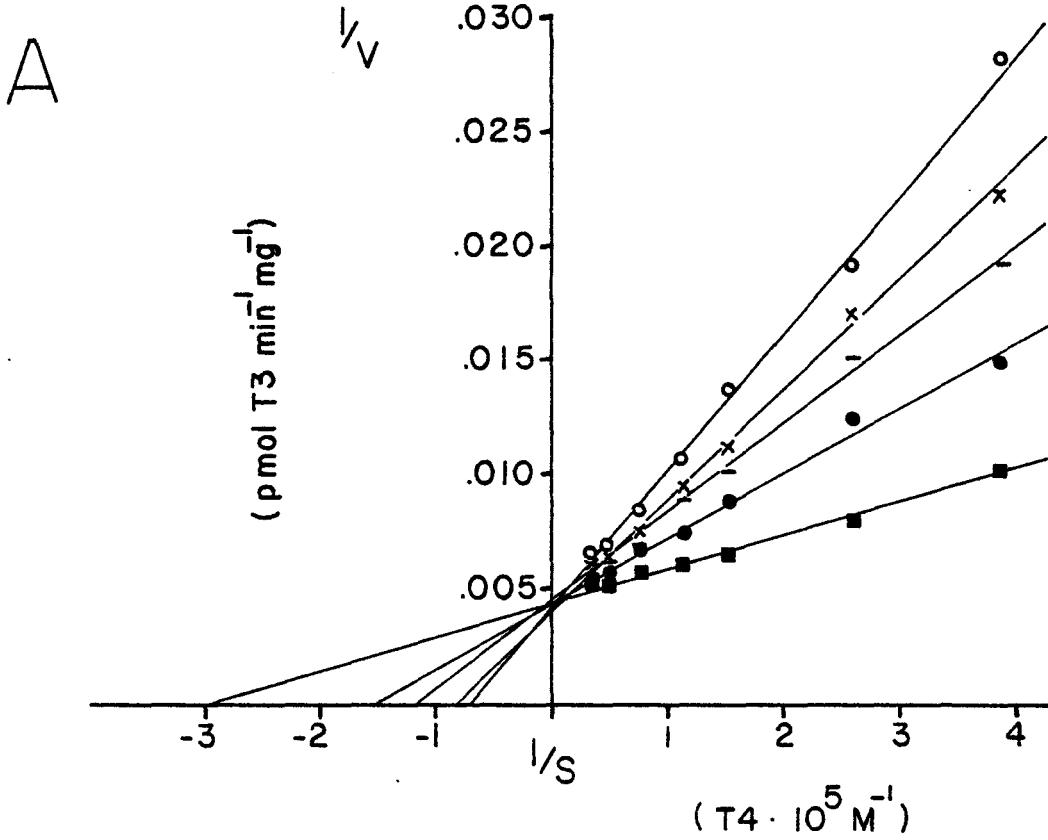
different concentrations of dithiothreitol (Figure 26). The apparent Km

for T4 varied from 1.03 μmol/L T4 at a concentration of 3 mmol/L dithio-

threitol (DTT) to 2.46 μmol/L T4 at a concentration of 12 mmol/L DTT.

The apparent maximum velocity (Vmax) was also dependent on DTT concen-

tration and varied from 65.8 pmol T3 $\text{min}^{-1}\text{mg}^{-1}$ at a concentration of 3

mmol/L DTT to 162 pmol T3 $\text{min}^{-1}\text{mg}^{-1}$ at a concentration of 12 mmol/L

DTT.

To determine the true Km for the Fraction III 5'-deiodinase at sub-

strate saturation for T4 and DTT, a double reciprocal plot of apparent Km

T4 values versus DTT concentration was plotted (Figure 27, A). A line

drawn through the apparent Km T4 values gave a y-intercept of (1/true Km

T4) and a x-intercept of (-1/true Km DTT) (Dixon and Webb, 1979a). The

true Km of T4 was calculated to be 5.0 μmol/L T4 and the true Km of DTT

was calculated to be 11.6 mmol/L DTT by this procedure. The true Vmax at

substrate saturation for T4 and DTT was determined in a similar manner by

a double reciprocal plot of apparent Vmax versus DTT concentration

(Figure 27, B) (Dixon and Webb, 1979a). The y-intercept of this plot in-

dicated that the true Vmax for iodothyronine 5'-deiodinase was 320 pmol

T3 min^{-1} mg^{-1}.

In a comparative study, the kinetic parameters of Fraction A (crude

homogenate), Fraction D (solubilized 5'-deiodinase), and Fraction III

(purified 5'-deiodinase) were determined for a constant 20 mmol/L concen-

tration of DTT with varying concentrations of T4. The velocity versus T4

concentration data were plotted in the form of a double reciprocal plot.

The apparent Km values for Fractions A, D, and III were determined from

the x-intercept of the primary plot to be: 4.25, 4.05, and 4.35 μmol/L

Figure 26. Lineweaver-Burk plot of data for the production of T3 from T4 by the Fraction III 5'-deiodinase at different concentrations of dithiothreitol (DTT).

Varying quantities of T4 were incubated with: o——o , 3 mmol/L DTT; •——• , 6 mmol/L DTT; □——□, 9 mmol/L DTT; and ■——■, 12 mmol/L DTT, and 100 µL of Fraction III (0.01 mg protein) in 0.15 $\underline{M}$ phosphate buffer (pH 6.5) for 30 minutes at 37 °C. The amount of T3 generated from the deiodination of T4 was quantitated by radioimmunoassay of an ethanol extract of the incubation mixture. For: 3, 6, 9, and 12 mmol/L DTT, the apparent K_m values were: 1.03, 1.70, 2.15, and 2.46 µmol/L T4, respectively and the apparent maximum velocities were: 73, 119, 152, and 182 pmol T3 min^{-1} mg^{-1}, respectively. The average slope (K_m/V_{max}) of the 4 parallel lines was 0.0142.
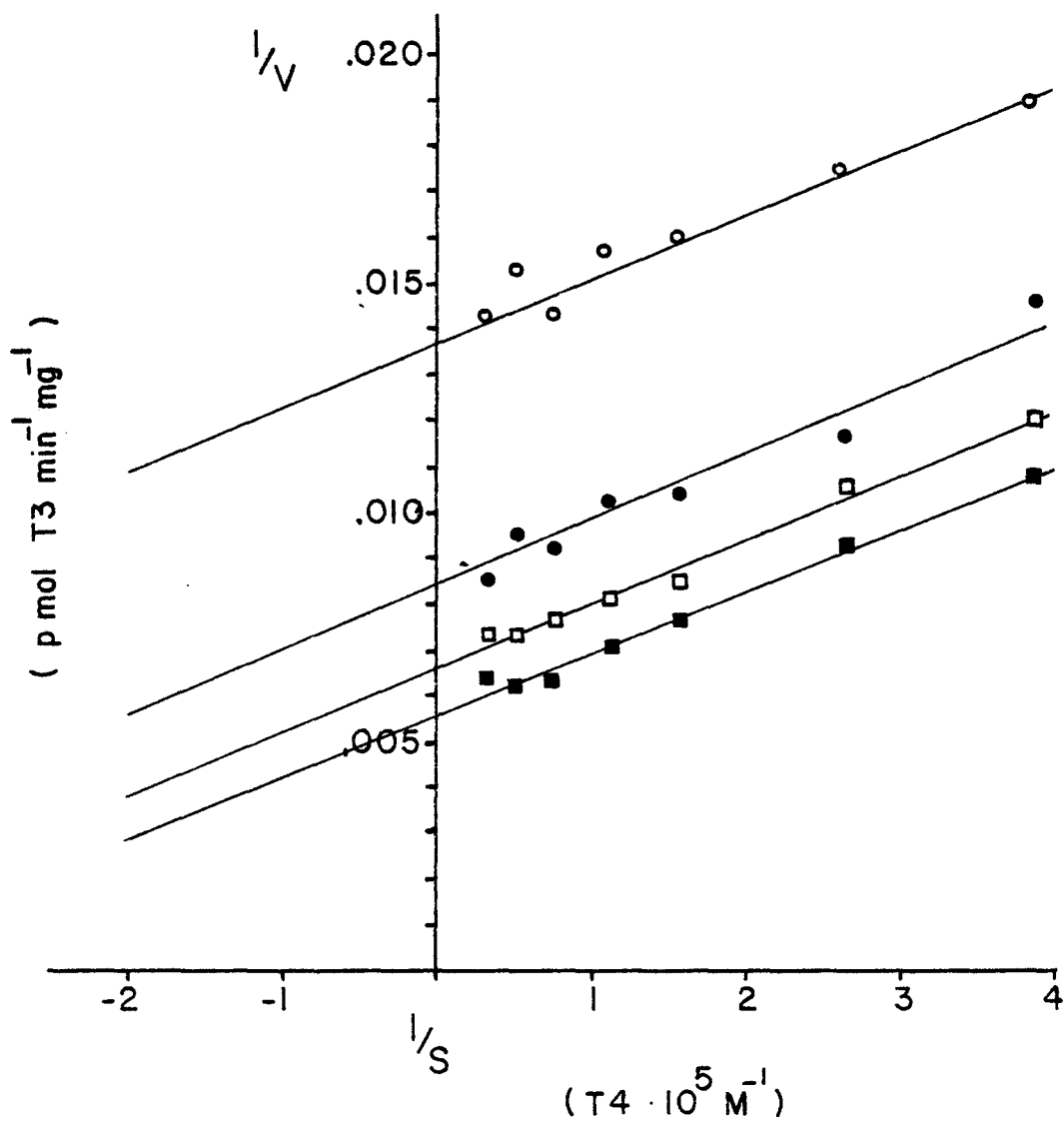
Figure 27. Determination of Km and Vmax values from replots of kinetic data.

A. A double reciprical plot of the apparent K_m values for T4 from Figure 26 was plotted with respect to dithiothreitol concentration. A line drawn through the 4 points had a slope (K_m DTT/K_m T4) of 2.297. The y-intercept (1/K_m T4) had a value of 2.0 x 10^5 M^{-1}, and the x-intercept (1/K_m DTT) had a value of -0.871. B. A double reciprical plot of the apparent V_{Max} values for the conversion of T4 to T3 from Figure 26 was plotted with respect to dithiothreitol concentration. A line drawn through the 4 points had a slope (K_m DTT/V_{Max}) of 0.0356. The y-intercept (1/V_{Max}) had a value of 0.00298, and the x-intercept (1/K_m DTT) had a value of -0.877. From the replots of kinetic data, the true K_m for T4, the true K_m for DTT, and the true V_{Max} for Fraction III were determined to be: 5.0 μmol/L T4, 11.4 mmol/L DTT, and 336 pmol T3 $min^{-1}mg^{-1}$, respectively.
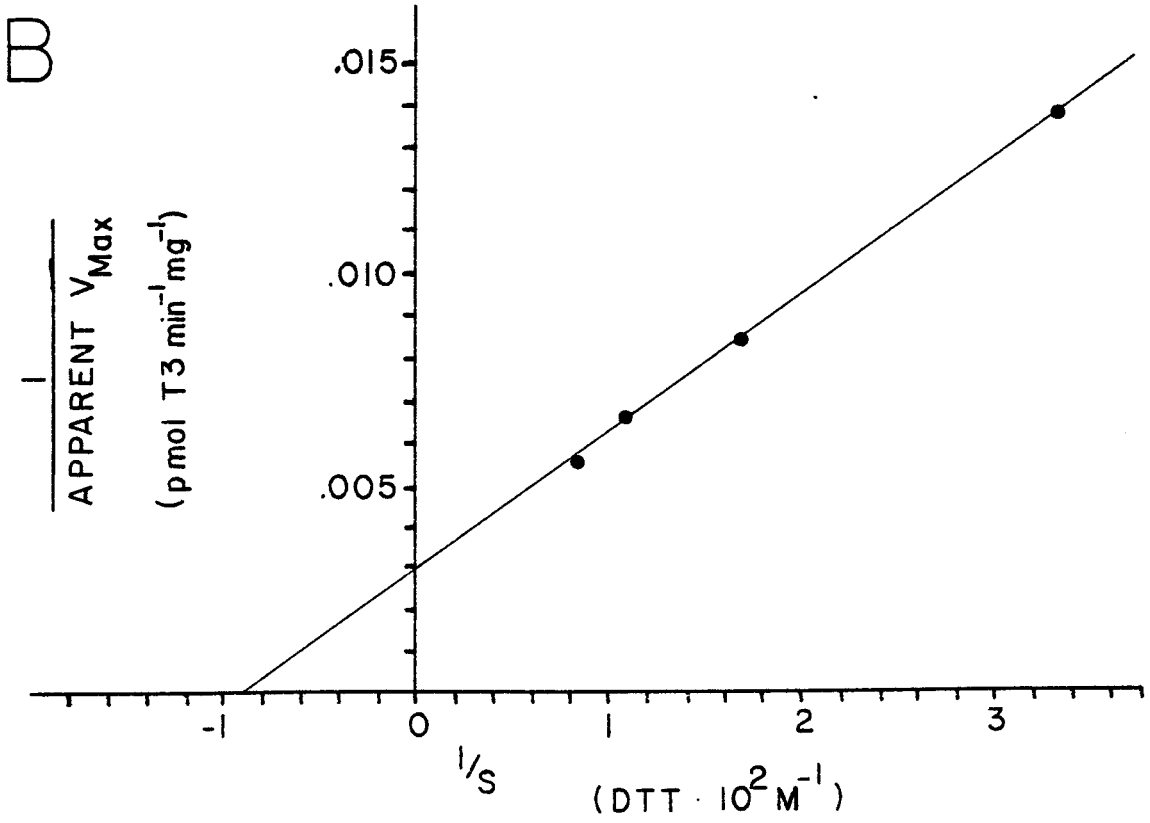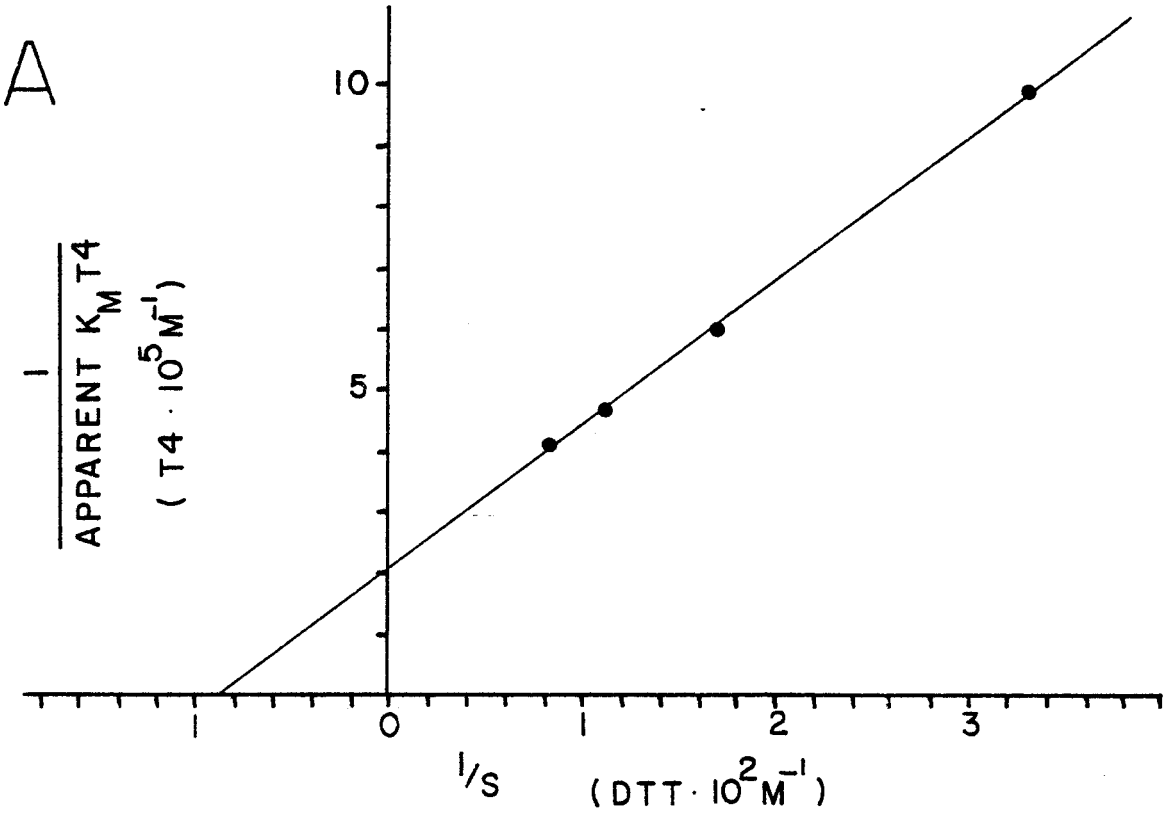
T4, respectively and the apparent Vmax values for Fractions A, D, and III were determined from the y-axis of the primary plot to be: 0.61, 1.42, and 285 pmol T3 $min^{-1}mg^{-1}$, respectively. These values represented comparative data for conditions approaching substrate saturation.

The fact that the Lineweaver-Burke plot gave parallel lines suggested that the reaction mechanism of the enzyme was that of a two-step transfer or ping-pong type reaction. Two additional studies were done to further investigate the possibility of a two-step transfer mechanism. In the first study (Figure 28), enzyme kinetics were performed in the presence of the competitive inhibitor, sodium salicylate. A double reciprocal plot of velocity versus T4 concentration was repeated. As observed in the absence of an inhibitor, parallel lines were obtained in the plots for DTT concentrations of 3, 6, and 12 mmol/L. In the second study (Figure 29), T4 and DTT concentrations were increased while a constant ratio of 2.6 μmol/L T4 : 1 mmol/L DTT was maintained. At this ratio, the velocity of Fraction III deiodinase increased in a linear manner over a range of from 1.3 to 32.2 μmol/L T4.

Isotopic Studies of The Deiodination of ^{125}I-T4 and ^{125}I-rT3

The effect of time and pH on the deiodination of ^{125}I-rT3 by Fraction D was studied as shown in Figure 30. Reverse T3 was rapidly metabolized by the crude enzyme preparation to a ^{125}I-labeled metabolite and free iodide (^{125}I) (Figure 30, A). Deiodination of rT3 was nearly complete after 30 minutes, however, the rT3 metabolite was further deiodinated with a corresponding increase in free iodide. The deiodination of rT3 by Fraction D occured more rapidly at pH 7.5 than at pH 7.0 or 6.5

Figure 28. Demonstration of parallel lines for the Lineweaver-Burk plot when kinetic data for the conversion of T4 to T3 was generated in the presence of a competitive inhibitor of T4.

　　　　Experimental detial was as described in Figure 26 with the exception that sodium salicylate (75 μmol/L) was added to the incubation mixture. □———□ , 3 mmol/L DTT; ○———○ , 6 mmol/L DTT; ●———● , 12 mmol/L DTT. The average slope of the 3 parallel lines was 0.0351.

$^1/_V$

$(\text{p mol T3 min}^{-1} \text{ mg}^{-1})$

.035

.030

.025

.020

.015

.010

.005

75 µM Sodium Salicylate

-2   -1   0   1   2   3   4

$^1/_S$ $(\text{T4} \cdot 10^5 \text{M}^{-1})$
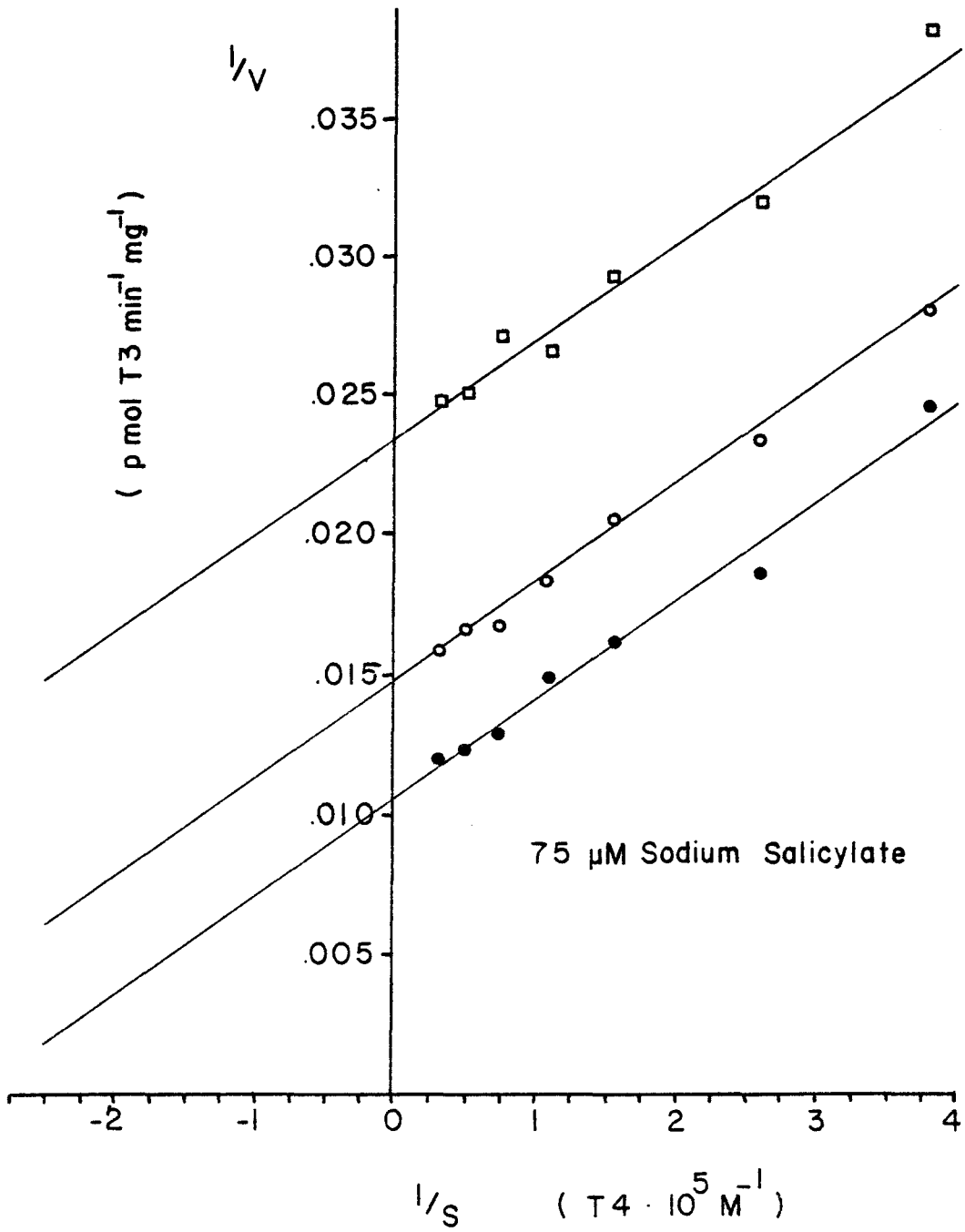
Figure 29. Linearity of iodothyronine 5'-deiodinase activity with in-
creasing T4 concentrations at a constant molar ratio of T4 to dithio-
threitol.

    Standard enzyme assay conditions for the incubation of Fraction III
with T4 were altered by varying the concentration of both T4 and dithio-
threitol. As concentrations of T4 were increased from 1.3 to 32.5
µmol/L, the concentration of dithiothreitol was increased from 0.5 to
12.5 mmol/L at a constant ratio of 2.6 µmol/L T4 to 1.0 mmol/L DTT. Data
are the mean ± standard deviation for quadruplicate determinations.
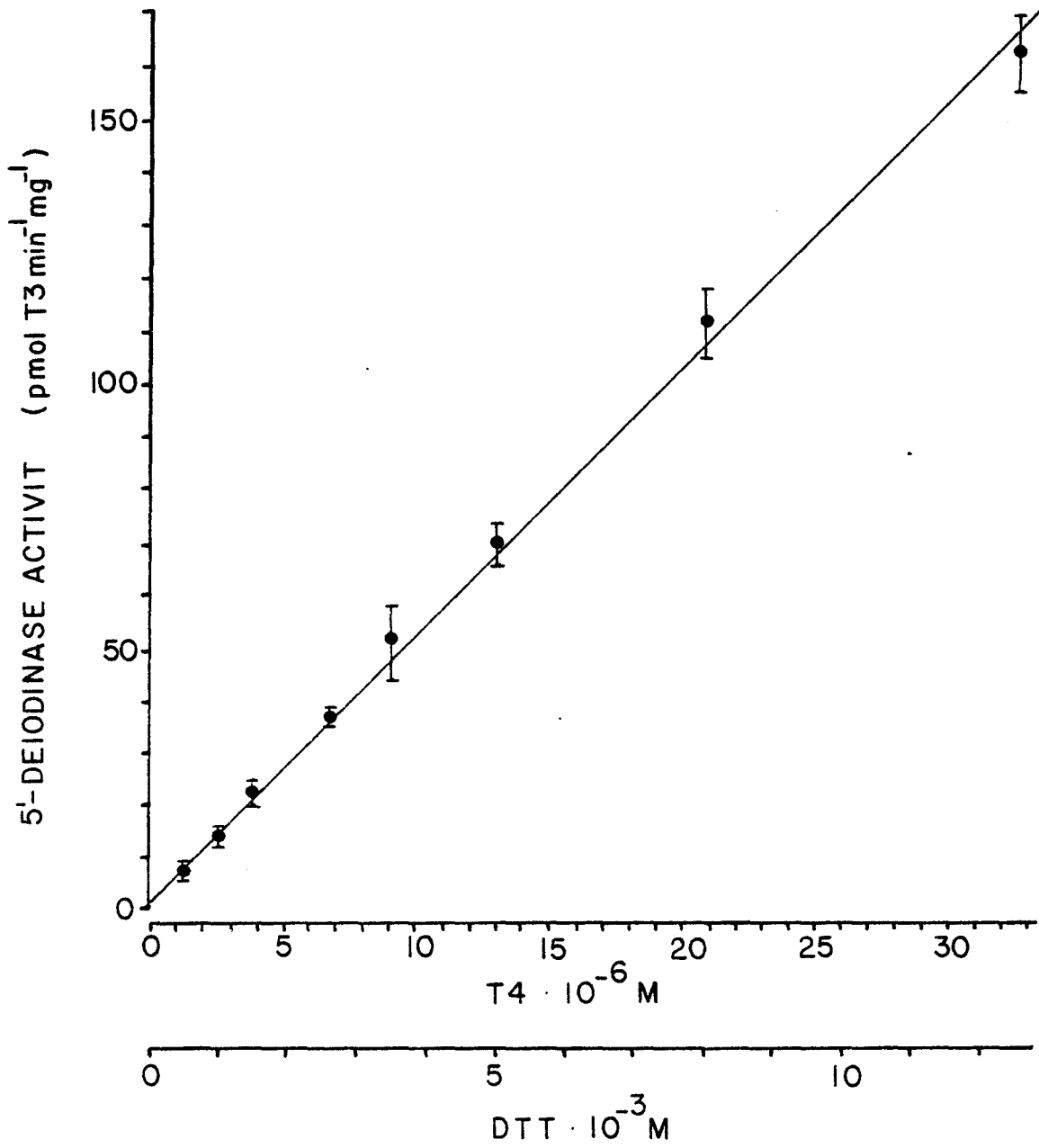
Figure 30. Effect of time and pH on the deiodination of rT3 by Fraction D.

100 μL of Fraction D containing 1.2 mg of protein was incubated for 0.60 min at 37 °C with 400 μL of a solution containing 15 μmol/L rT3, 9.2 μCi ^{125}I-rT3, 20 mmol/L dithiothreitol, and 100 mmol/L phosphate buffer, pH 6.5-7.5. The enzyme assay was terminated by adding 50 μL of a solution containing 2 mol NaOH and 5 mol NaCl. Chromatography of 0.4 mL of the alkaline extract on Sephadex G-25 sf (See Materials and Methods) resulted in 3 peaks of radioactivity: free iodide (^{125}I), fractions 8-11; ^{125}I-rT3 metabolite, fractions 14-20; and rT3 (^{125}I-rT3), fractions 22-30. The results are expressed in terms of percent of total counts (^{125}I) for each peak. A. Fraction D was incubated for: 0, 15, 30, or 60 minutes in phosphate buffer, pH 7.5. B. Fraction D was incubated for 30 minutes in phosphate buffer, pH 6.5, 7.0, or 7.5.
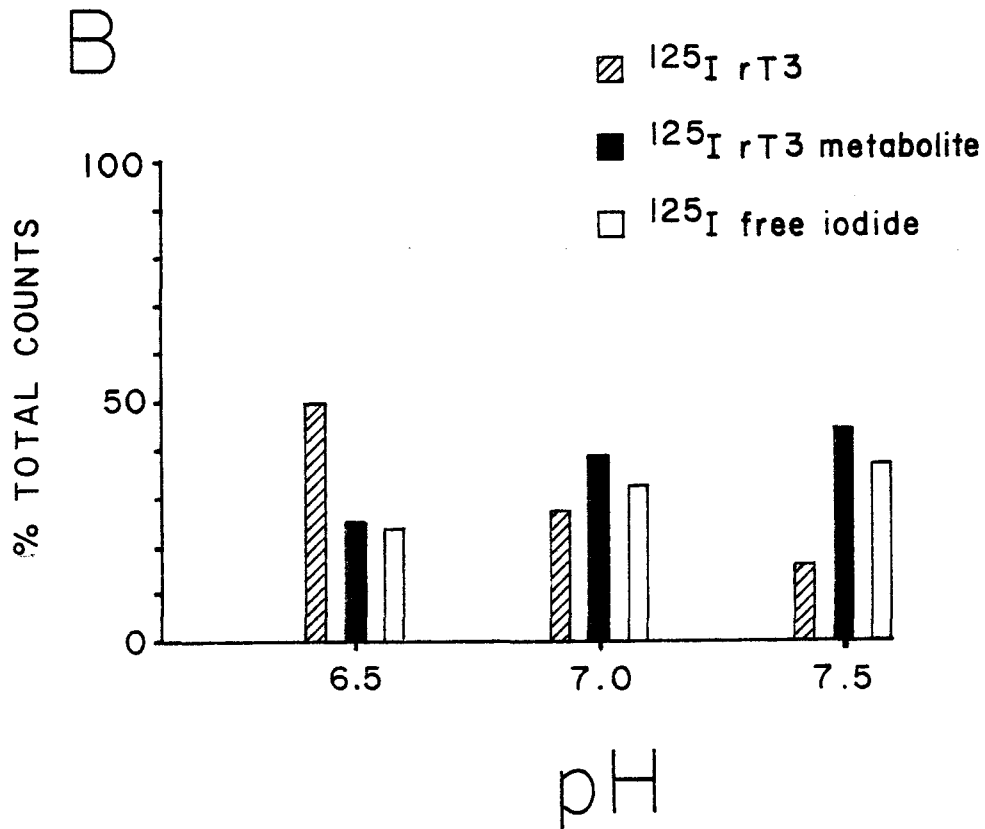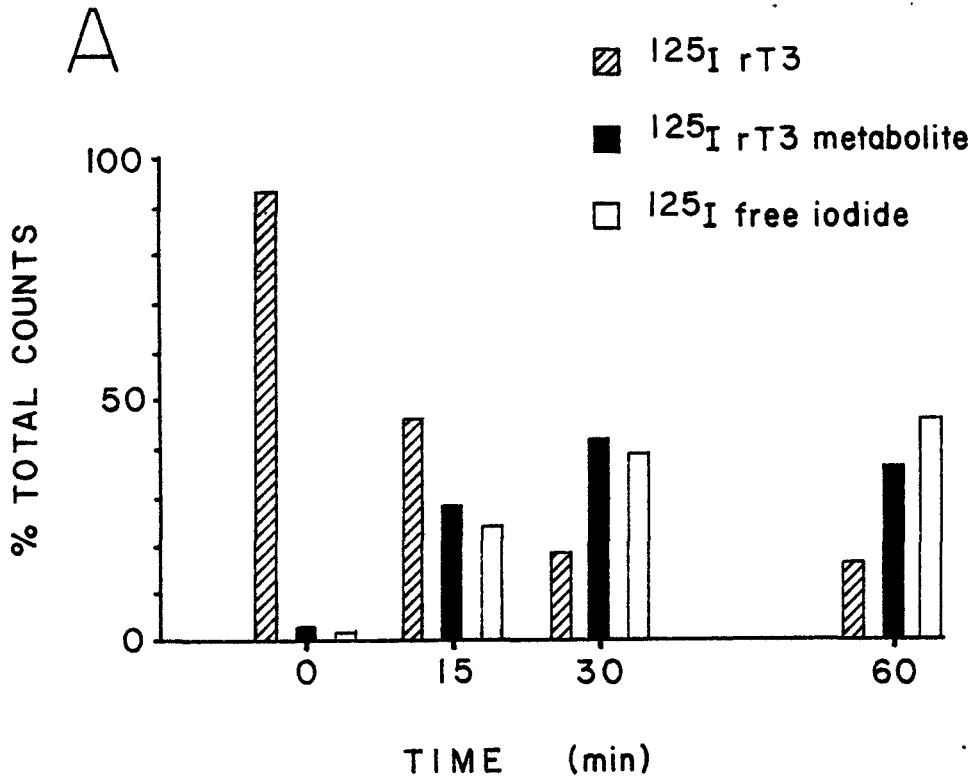
(Figure 30, B).

The rate of deiodination of ^{125}I-rT3 with time (Figure 31) and of ^{125}I-T4 with time (Figure 32) was determined for Fraction D, Fraction III and for a mixture of Fraction D and Fraction III. The results after 30 minutes and 60 minutes for the deiodination of rT3 and T4, respectively are presented in Table 6.

During a 30 minute incubation at pH 7.5, Fraction III deiodinated approximately 4 % of the rT3 substrate (Figure 31, A). Fraction D deiodinated approximately 72 % of the rT3 substrate in the same time period (Figure 31, B). During a 60 minute incubation at pH 6.5, Fraction III and Fraction D deiodinated approximately 8 % (Figure 32, A) and 4 % (Figure 32, B) of the T4 substrate, respectively. Therefore, despite the 2-fold greater activity of Fraction III in the deiodination of T4, Fraction III was 18-fold less active than Fraction D in the deiodination of rT3.

When the enzyme assay was run with a mixture containing equal portions of Fraction III and Fraction D, the rate of deiodination of rT3 was equal to approximately one half that of Fraction D alone (Figure 39, C). When the mixture of Fraction D and Fraction III was assayed for the deiodination of T4, the rate was approximately 1/3 greater than that of Fraction D alone (Figure 32, C).

Figure 31. Comparison of iodothyronine 5'-deiodinase activity in Fraction III, Fraction D, and a mixture of Fraction D plus Fraction III for the deiodination of ^{125}I-rT3.

Experimental details were as described in Figure 30. A. 100 μL Fraction III containing 10 μg protein was incubated for 0, 15, 30 and 60 minutes in phosphate buffer, pH 7.5. B. 100 μL Fraction D containing 1.2 mg protein was incubated for 0, 15, 30 and 60 minutes in phosphate buffer, pH 7.5. C. A mixture of 50 μL Fraction D and 50 μL Fraction III was incubated for 0, 15, 30, and 60 minutes in phosphate buffer, pH 7.5. The amount of radioactivity in each of 3 peaks following Sephadex G-25 sf chromatography was expressed as a percentage of total counts: ●——● , ^{125}I-rT3; ○——○, free ^{125}I; □——□ , ^{125}I-labeled rT3 metabolite.

Figure 32. Comparison of iodothyronine 5'-deiodinase activity in Fraction III, Fraction D, and a mixture of Fraction D plus Fraction III for the conversion of T4 to T3.

100 µL samples containing: A. Fraction III (10 µg protein) B. Fraction D (1.2 mg protein) or C. 50:50 mixture of Fraction D plus Fraction III were incubated for: 0, 30 and 60 minute time intervals at 37 °C with 400 µL of a solution.contianing 15 µmol/L T4, 57.2 µCi ^{125}I-T4, 20 mmol/L dithiothreitol and 100 mmol/L phosphate buffer, pH 6.5. The enzyme assay was terminated by adding 50 µL of a solution containing 2 mol/L NaOH and 5 mol/L NaCl. Chromatography of 0.4 mL of the alkaline extract on Sephadex G-25 sf (See Materials and Methods) resulted in 3 peaks of radioactivity: o——o, free iodide (^{125}I), fractions 8-11; □——□, ^{125}I-T3, fractions 14-20; and ▲——▲, ^{125}I-T4, fractions 22-30. The amount of radioactiv-ity in each peak was expressed as a percentage of total count.
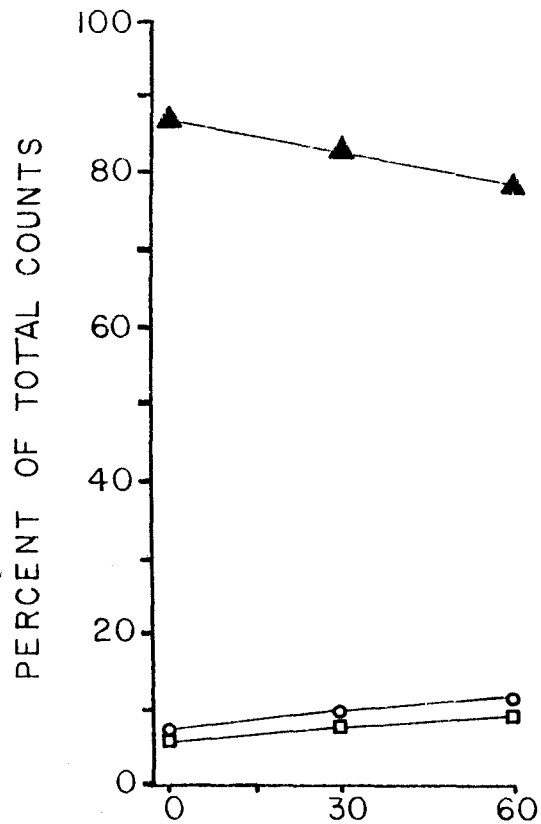
A FRACTION III B FRACTION D C FRACTION D+III

PERCENT OF TOTAL COUNTS
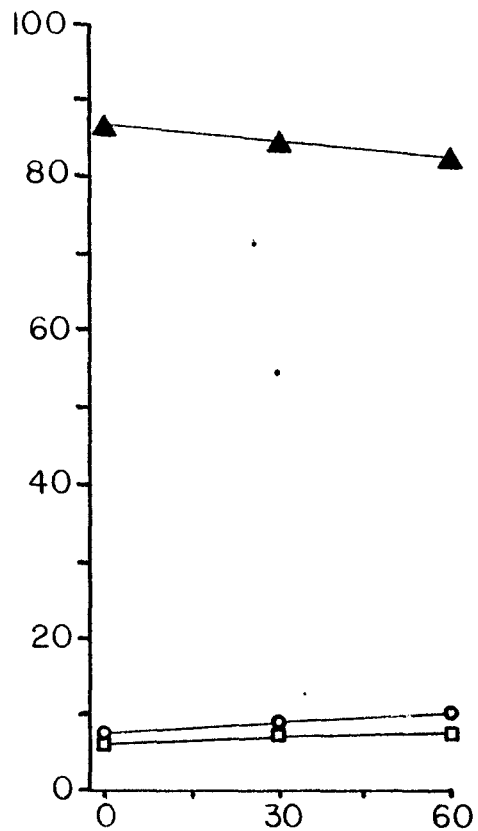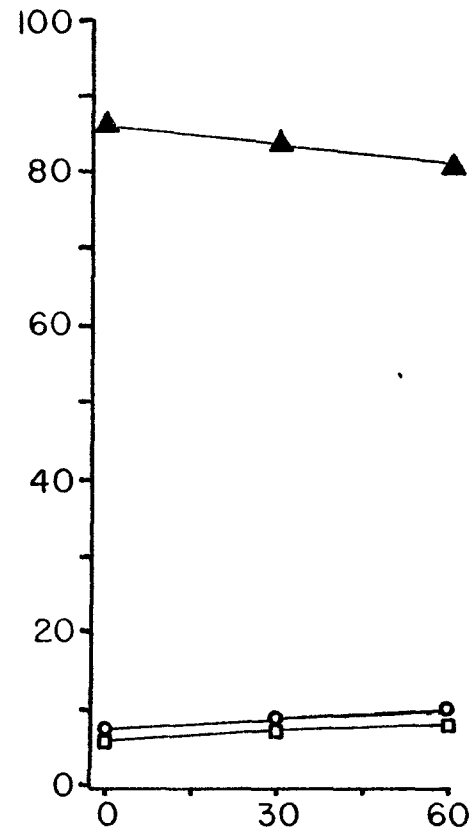
TIME. (min)

Table 6

Summary of Results (Figures 31 and 32) For the Deiodination of $^{125}I-rT3$

and $^{125}I-T4$ BY Fraction D and Fraction III.

SAMPLE	DEIODINATION OF $^{125}I-rT3$				DEIODINATION OF $^{125}I-T4$			
	LABEL	PERCENT OF TOTAL COUNTS			LABEL	PERCENT OF TOTAL COUNTS		
		0 min	30 min	Δ %		0 min	60 min	Δ %
Fraction III	$^{125}I-rT3$	94.0	90.0	− 4.0	$^{125}I-T4$	86.9	78.8	−8.1
	$^{125}I-rT3$ metabolite	2.1	3.8	+ 1.7	$^{125}I-T3$	6.1	9.4	+3.3
	Free ^{125}I	3.9	6.0	+ 2.1	Free ^{125}I	6.9	11.6	+4.7
Fraction D	$^{125}I-rT3$	94.2	21.8	−72.4	$^{125}I-T4$	86.6	82.6	−4.0
	$^{125}I-rT3$ metabolite	3.6	40.0	+36.4	$^{125}I-T3$	6.1	6.1	+1.3
	Free ^{125}I	2.2	38.2	+36.2	Free ^{125}I	7.2	9.8	+2.6
Fraction III	$^{125}I-rT3$	94.0	61.5	−32.5	$^{125}I-T4$	86.3	81.0	−5.3
&	$^{125}I-rT3$ metabolite	3.9	20.3	+16.4	$^{125}I-T3$	6.3	8.4	+2.1
Fraction D	Free ^{125}I	2.0	18.0	+16.0	Free ^{125}I	7.2	10.4	+3.2

CHAPTER IV

DISCUSSION

The enzyme catalyzed conversion of T4 to T3 has been demonstrated in a number of mammalian tissues (Chopra, 1977a; Cheron et al, 1980; Ishii et al, 1981), and has been implicated as the major source of serum T3 in man (Braverman et al., 1970; Chopra, 1978). The conversion of T4 to T3 involves the removal of a single iodine atom from the C_5', position of the outer iodothyronine ring and, therefore, the converting enzyme belongs to a class of enzymes called iodothyronine 5'-deiodinases. Other deiodinases which remove an iodine from the inner iodothyronine ring belong to a second, distinct class of enzymes, the iodothyronine 5-deiodinases (Cavalieri et al., 1977; Kaplan and Utiger, 1978; Gavin et al., 1980). The present study has delt exclusively with the 5'-deiodinase whose substrates include thyronines iodinated in the 3'-and/or 5'-positions, and more specifically, with the isolation and characterization of the rat liver 5'-deiodinase that converts T4 to T3.

After a conventional subcellular fractionation of rat liver homogenates, the T4 5'-deiodinase was found to be associated with cellular membranes rather than cytosolic proteins, and in line with previous studies using both rat liver and kidney homogenates (Visser et al., 1976b; Takaishi et al., 1979; Maciel et al., 1979), the greatest yield of thyroxine 5'-deiodinase activity was obtained in the crude microsomal preparation. The crude microsomal fraction, however, contained an average of no more than 50 % of the total activity, and an increase in specific

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activity of no more than 4-fold. The majority of the remaining activity was located in the crude nuclear fraction. Upon further subfractionation of the crude microsomal and nuclear fractions, it was discovered that in addition to the microsomes, the plasma membrane was also an important source of rat liver 5'-deiodinase activity. Therefore, rather than using a microsomal preparation as used in previous studies (Takashi et al., 1979; Leonard and Rosenberg, 1980a; Tseng and Latham, 1981), or a plasma membrane preparation, a cellular membrane preparation containing both microsomal and plasma membranes was chosen as the starting material for solubilization and purification of the 5'-deiodinase. This decision was made due to the fact that a substantial purification of the enzyme was not achieved by subcellular fractionation.

The solubilization procedure used in this study was effective in selectively extracting thyroxine 5'-deiodinase activity from the crude rat liver membrane preparation. The yield of solubilized 5'-deiodinase activity (~60 %) following extraction with Nonidet P-40 was comparable to that reported previously (60-70 %) for extraction with deoxycholate (Leonard and Rosenberg, 1980a), and the 0.05 -0.7 % Nonidet P-40 extract provided a 1.4-fold purification of the crude membrane preparation. Nonidet P-40 rather than deoxycholate, as used in previous studies (Takaishi et al., 1979; Leonard and Rosenberg, 1980a; 1980b; Tseng and Latham, 1981), was chosen for solubilization of the rat liver 5'-deiodinase because the catalytic activity of the enzyme was much less inhibited in the presence of Nonidet P-40 than in the presence of deoxycholate (See Figure 18). As a result, enzyme assays could be carried out in the presence of low levels of Nonidet P-40 without the necessity of detergent removal. An

additional advantage of the use of the nonionic detergent was that losses of enzyme activity during a single pass through a Sepharose 6B gel infiltration column were in the range of 35-45 % when Nonidet P-40 was used as the dispersing agent compared to losses in activity of greater than 90 % when deoxycholate was used as the dispersing agent (Leonard and Rosenberg, 1980a).

The extracted rat liver 5'-deiodinase remained in the supernatant solution after high speed centrifugation (78,000 x g, 60 min) and, therefore, appeared to be soluble. The enzyme precipitated following acidification (pH 5.8 -6.0), however, in a manner characteristic of detergent "solubilized" lipoprotein complexes (Penefsky and Tzagoloff, 1971). The lipoprotein nature of the Nonidet P-40 solubilized 5'-deiodinase was further supported by demonstrating that the enzyme was poorly soluble in aqueous buffer. Thus, while Sepharose 6B-CL chromatography in the presence of 0.1% Nonidet P-40 allowed elution of the 5'-deiodinase after the void volume, chromatography in the absence of surfactant resulted in the formation of macromolecular complexes of the enzyme which eluted in the void volume. A similar phenomenon was also reported for the thyroxine 5'-deiodinase extracted from rat liver microsomal preparations with deoxycholate (Leonard and Rosenberg, 1980a). In addition, evidence of the intrinsic nature of the 5'-deiodinase, as demonstrated by the fact that extraction required extensive membrane disassembly (Figures 6 and 7), indicated that the enzyme had a strong association with membrane lipids (Takaishi et al., 1979). Therefore, rather than existing in true solution, the Nonidet P-40 extracted 5'-deiodinase was apparently suspended in detergent micelles containing the 5'-deiodinase and associated mem-

brane phospholipids.

The presence of residual amounts of membrane phospholipid has been reported to be a requirement of enzymatic activity for the rat liver 5'-deiodinase (Leonard and Rosenberg, 1980a). Therefore, delipidation which is known to occur in the presence of surfactants during membrane solubilization (Tzagoloff and Penefsky, 1971) and gel filtration chromatography (Fry and Green, 1980; Penefsky and Tzagoloff, 1971) could explain, in part, losses of activity whch occured in the present study. Accordingly, optimum conditions for solubilization and purification of the 5'-deiodinase were empirically determined to maximize stability of the extracted enzyme complex. In the present study, the use of Nonidet P-40 allowed for the first time the solubilization of an active form of the 5'-deiodinase complex from rat liver membranes which was reasonably stable in low concentrations of the dispersing detergent.

Preliminary attempts to purify the 5'-deiodinase demonstrated the usefulness of DEAE cellulose, hydroxylapatite, and Sepharose 6B-CL chroma-tography steps in the purification of the Nonidet P-40 solubilized enzyme. High yields and increases in relative specific activity following each step supported the efficacy of the multistep procedure. The first step in the purification scheme involved reverse anion exchange chromatography on DEAE cellulose. The majority of the 5'-deiodinase did not adsorb to the column matrix and was, therefore, separated from the proteins that did adsorb. In addition, elution of the 5'-deiodinase from the cellulose column was retarded, allowing separation of the enzyme from the bulk of the non-adsorbed protein also. As a result, the bulk of the cellulose step allowed purification of the 5'-deiodinase through both ion

exchange interactions and apparent hydrophobic interactions in a single step. The second step involved adsorption chromatography on hydroxylapatite. The third step, gel filtration chromatography on Sepharose 6B-CL, permitted the removal of high and low molecular weight contaminants. The enzyme was remarkably stable through all stages of purification. Surprisingly, the first two steps in the purification scheme resulted in a mild activation of the enzyme. This behavior was very similar to the enhancement of activity following solubilization with deoxycholate (Takaishi et. al., 1979) and may have been associated with the removal of tissue factors which mask enzymatic activity in crude homogenate preparations (Leonard and Rosenberg, 1977).

Purification of the rat liver iodothyronine 5'-deiodinase by the method reported here resulted in a 580-fold enrichment in specific activity and a 24 % recovery. The final product was purified from 100 to 300 times greater than microsomal preparations previously used for characterization of the 5'-deiodinase, which had increases in specific activity of from 2- to 6-fold (Visser et. al., 1976b; Hoffken et. al., 1978; auf dem Brinke et. al., 1979). An additional advantage of the purification scheme was that the final product was prepared in a soluble, yet active form. Therefore, detergent removal was not required prior to performing kinetic determinations. All previous kinetic studies have been performed on insoluble enzyme preparations of either cellular membranes or DOC-free extracts. The purification procedure used in this study has for the first time allowed kinetic assays to be performed with a soluble enzyme preparation.

Upon gel filtration chromatography of the purified 5'-deiodinase, a

single sharp peak of activity was observed with an apparent molecular weight of approximately 250,000 and an apparent Stokes' radius of approximately 5.8 nm. There was no detectable activity at the void volume and there were no other minor peaks of activity. Based on results with deoxycholate solubilized enzyme preparations, where two enzyme forms of molecular weight 85,000-95,000 and 200,000 were observed (Leonard and Rosenberg, 1980a; Tseng and Latham, 1981), the Nonidet P-40 solubilized 5'-deiodinase complex may have been a dimer. The presence of a monomeric subunit of the 5'-deiodinase may, therefore, have been the cause of the shoulder in the low molecular weight range during chromatography of Fraction II on Sepharose 6B-CL (Figure 11). The difference in the molecular weight estimates of 250,000 in the present study and 200,000 in the previous study was expected on the basis that the nonionic detergents, Triton X-100 and Nonidet P-40 tend to form larger mixed micelles with membrane proteins than do anionic detergents (Helenius and Simons, 1975).

Column isoelectric focusing of the purified iodothyronine 5'-deiodinase resulted in the formation of a single major peak of enzyme activity which had a pI of 7.4. The presence of a single peak of activity indicated that the majority of the Nonidet P-40 solubilized, enzyme complex existed in a single electrophorectic form. The formation of minor peaks of activity were not consistent in size or location and, therefore, were not considered to be evidence of multiple isoenzyme forms of the 5'-deiodinase. Approximately 10 % or less of the applied activity was recovered due to precipitation of the enzyme despite the presence of 0.1 % Nonidet P-40. Therefore, isoelectric focusing was not included as

a preparatory step in the purification scheme. The procedure would be advantageous, however, in cases where a high degree of purity is of paramount importance.

The catalytic activity of the purified iodothyronine 5'-deiodinase was studied under a variety of conditions to determine the parameters that effect the in vitro conversion of T4 to T3. It was observed that factors modulating the 5'-deiodination of T4 included the temperature and pH of incubation, that activity was linear with respect to protein concentration, and that activity was dependent on the concentration of substrate (T4). These effects demonstrated that the converting activity of the Fraction III preparation was enzymatic in nature.

Fraction III iodothyronine 5'-deiodinase activity had an absolute requirement for sulfhydryl group-containing compounds. Unlike crude homogenates which were active in the absence of exogenous cofactor (Chopra, 1977b; Balsam and Ingbar, 1979; Harris et. al., 1979), the catalytic activity of the purified enzyme required the presence of a reduced thiol. The relative potency of several selected sulfhydryl compounds is shown in Figure 19. Dithiothreitol had the greatest stimulatory effect, demonstrating substracre saturation above a 20 mmol/L concentration. Beta-mercaptoethanol, reduced glutathione, cysteine and lipoamide also had concentration dependent stimulatory effects. Due to the fact that the thiol group was an essential cofactor, it was considered to have the status of a second substrate.

The T4 to T3 converting activity of the purified 5'-deiodinase demonstrated no apparent requirement for the presence of metal cofactors or for the presence of other monovalent or divalent ions. In line with

results from previous studies (Visser et. al., 1976b; Chopra, 1976; Sorimachi and Robbins, 1979), EDTA had no effect and Ca^{2+}, Mg^{2+}, and Mn^{2+} at 1 mmol/L concentrations had no effect or only slightly increased the rate of deiodination. Ferric ion did not stimulate Fraction III activity, however, ferric ion did accelerate the nonenzymatic deiodination of T4 in blank tubes containing heat inactivated enzyme. Therefore, it was concluded that the stimulation of T4 to T3 conversion by ferric ion reported previously (Chiraseveenuprapund et. al., 1978) may have been the result of chemical rather than enzymatic catalysis. In the present study, 0.1 mmol/L Zn^{2+} inhibited 5'-deiodinase activity, but it is not known whether this effect was on the enzyme, on the cofactor, or on the solubility of the substrate.

Mercury and lead had a potent inhibitory effect on the T4 to T3 converting activity which could be reversed with the addition of dithiothreitol to the incubation mixture. Due to the catalytic requirement of the 5'-deiodinase for a thiol cofactor, this effect may have been due to a sulfhydryl-blocking effect on the cofactor (Chopra, 1977a). On the other hand, it has also been demonstrated that the enzyme itself has an essential sulfhydryl group in its active site (Leonard and Rosenberg, 1980b). Therefore, the inhibition by heavy metals may also have been due in part to a direct inhibition of the enzyme. In either case, the inhibition by mercury and lead was competitive in nature and could be overcome with the addition of dithiothreitol.

Inhibition of 5'-deiodinase activity by bovine serum albumin and by human serum proteins was observed in the present study as well as in a previous report (Chiraseveenuprapund et. al., 1978). The nature of the

inhibition was presumed to be due to the thyroxine binding capacity of albumin and thyroid binding globulin which are reported to have association constants for thyroxine of 1×10^6 mol^{-1} and 3×10^{10} mol^{-1}, respectively (Nicoloff, 1978). In a likewise manner, rat liver and kidney membrane proteins which have high affinities for T4 (Oppenheimer and Surks, 1975; Sterling et. al., 1977; Rall, 1978) have the potential for interfering with the kinetics of 5'-deiodinase activity by lowering the effective substrate concentration. Therefore, extensive purification of the enzyme activity is highly desirable prior to the determination of kinetic parameters.

In addition to the agents mentioned above, the present study included an examination of the effects of several compounds which have been reported to inhibit either the thyroidal or peripheral metabolism of thyroxine. Of the compounds tested, the most potent inhibitor of Fraction III activity was reverse T3. Preliminary kinetic data from two previous studies utilizing the crude rat liver homogenate preparations as the source of 5'-deiodinase activity indicated that rT3 inhibited the 5'-deiodinase in an apparent competitive manner with respect to T4 (Chopra, 1977a; Kaplan and Utiger, 1978) and, therefore, that T4 and rT3 compete for the same binding site of the 5'-deiodinase. On the other hand, my results with the purified Fraction III enzyme suggest non-competitive inhibition by rT3. Non-competitive inhibition suggests that rT3 binds to a site on the enzyme other than that for the binding of T4 and dithiothreitol and, therefore, that the purified thyroxine 5'-deiodinase has a minimum of two binding sites for iodothyronines. A similar conclusion was reached by Hüfner et. al., (1977) in a report in which kinetic stu-

dies were performed with rat liver homogenates.

Inhibition of thyroxine 5'-deiodinase activity by propylthiouracil (PTU) was observed to occur in the present study and in a previous study (Chopra, 1977a) in an apparent uncompetitive manner. This type of inhibition is characteristic of an inhibitor that binds reversibly to an enzyme-substrate complex but not to the free enzyme. With regard to the 5'-deiodinase, a likely candidate for an enzyme-substrate complex that would have a high affinity for PTU is the enzyme-sulfenyl iodide intermediate which has been postulated to result from the deiodination of T4 (Visser et. al., 1979b; Leonard and Rosenberg, 1980b). This hypothesis is supported by the work of Cunningham, who has shown that PTU readily forms mixed disulfides with protein-sulphenyl iodide groups (Cunningham, 1964). It has also been shown that uracil without the sulfur component is devoid of inhibitory activity (Visser et. al., 1979b) and, therefore, that the affinity of PTU for the enzyme was derived solely from the presence of the thiol group. Additionally, it was observed that PTU inhibited the 5'-deiodinase in a competitive manner with respect to dithiothreitol. The competitive nature of the inhibition by PTU could be explained as a competition between dithiothreitol and PTU for the postulated sulphenyl iodide group. The data obtained in the present study, therefore, indicate that the 5'-deiodination of T4 was very similar to the 5'-deiodination of rT3 which also was inhibited by PTU in an uncompetitive manner with respect to rT3 and in a competitive manner with respect to dithiothreitol (Visser et. al., 1979a).

Other compounds were shown to inhibit the Fraction III 5'-deiodinase in a competitive manner. Iodipamide (Cholografin), an iodi-

enzyme competitively with respect to both T4 and dithiothreitol. Other iodinated x-ray contrast agents such as iopidate (Oragrafin), iopanoic acid (Telepaque) and sodium tyropanoate (Bilopaque) have also been shown to be potent dose dependent inhibitors of the T4 to T3 converting enzyme in rat liver (Kaplan and Utiger, 1978; Chopra et. al., 1978) and pituitary homogenates (Larsen et. al., 1979), and Oragrafin, like Cholografin, has been shown to be a competitive inhibitor with respect to T4 (Chopra et. al., 1978). The x-ray contrast agents were structurally related to the idothyronines in that they all contained a saturated phenol ring containing iodine substituant. Sodium diatrizoate (Hypaque), on the other hand, did not have an inhibitory effect on the purified 5'-deiodinase despite a similarity in structure to the above mentioned x-ray contrast agents. This may have been due to charge repulsion or to steric hindrance.

Propranolol, a beta-adrenergic blocking agent commonly used in the treatment of hyperthyroid patients, also demonstrated competitive inhibition in a dose dependent manner with respect to both T4 and dithiothreitol. Binding affinity was poor, however, and a 2 mmol/L concentration of propranolol was required to inhibit activity by 50 %. Previous studies (Lumholtz et. al., 1979; van Noordan et. al., 1979) have also demonstrated the in vitro inhibition of 5'-deiodinase activity by propranolol, but failed to demonstrate a concentration dependent inhibitory effect in their crude enzyme preparations. Two other drugs used to treat hyperthyroid patients: dexamethasone, which has been shown to decrease the in vivo conversion of T4 to T3, (Croxson et. al., 1977; Chopra et. al., 1975), and methimazole, an antithyroid agent, (Chira-

seveenuprapund et. al., 1978) were found not to have an inhibitory effect on the purified 5'-deiodinase. These results were also observed in some other recent studies which employed rat liver (Chopra, 1977a) and kidney homogenates (Chiraseveenprapund et. al., 1978).

Sodium salicylate and anilino-naphthalenesulfonic acid (ANS) have been known for some time to displace T4 and T3 from circulating thyroxine binding proteins. More recently, it has been shown that salicylate and ANS also inhibit the T4 to T3 converting activity of the 5'-deiodinase (Chiraseveenuprapund et. al., 1978; Chopra et. al., 1980). Therefore, while the possibility exists that salicylate and ANS may compete with T4 for binding sites of the 5'-deiodinase as well as for binding sites of the transport proteins, these observations have also been interpreted to indicate that a protein-bound form of T4 could be the normal substrate for the T4 to T3 converting enzyme (Chiraseveenuprapund et. al., 1978). The protein bound to T4 in this case would be an intracellular protein, not thyroid binding globulin (TBG). In the present study, the fact that removal of the soluble proteins (Fraction B) from the membrane preparation (Fraction C) did not result in the loss of enzymatic activity and the fact that the presence of BSA and human serum strongly inhibited the catalytic rate strongly suggest that the free form of thyroxine was the normal substrate for the 5'-deiodinase.

Michaelis-Menten kinetics were observed in the present study for the conversion of T4 to T3 by the Fraction III 5'-deiodinase when enzyme assays were carried out in the presence of 0.2 % Nonidet P-40. Thus, when kinetic data was plotted in a double reciprical manner for velocity versus substrate concentration, the data was linear for the range of:

1 to 20 μmol/L T4 and 1 to 20 mmol/L dithiothreitol. Similar results were observed for kinetic assays carried out with crude rat liver preparations (Hüfner et. al., 1977; Chopra, 1977a; Kaplan and Utiger, 1978; Visser, 1979) and crude rat kidney preparations (Leonard and Rosenberg, 1978). The apparent Km (4.35 μmol/L T4) determined for the purified Fraction III 5'-deiodinase was in accordance with values previously reported for crude preparations of the T4 5'-deiodinase (0.8 to 7.7 μmol/L T4) (Hüfner et. al., 1977; Chopra, 1977a; Kaplan and Utiger, 1978; Leonard and Rosenberg, 1978; auf dem Brinke et. al., 1979; Visser et. al., 1979a) and was approximately equal to the apparent Km of the Fraction A 5'-deiodinase (4.25 μmol/L T4). The close relationship of the Km of the purified enzyme to that of the crude membrane bound preparation demonstrated that the structural integrity of the detergent solubilized enzyme had not been greatly altered during solubilization and purification.

The magnitude of T3 generation from T4 by the Fraction III iodothyronine 5'-deiodinase observed in the present study was approximately 300 pmol T3 $min^{-1}mg^{-1}$. This result reflects the increased purity of the Fraction III preparation as compared to the rat liver microsomal preparation (Fraction P) and the crude rat liver homogenate (Fraction A) which had velocities of 1.81 pmol T3 $min^{-1}mg^{-1}$ and 0.53 pmol T3 $min^{-1}mg^{-1}$, respectively. These results approximated previously reported velocities of: 0.04 pmol T3 $min^{-1}mg^{-1}$ (Hüfner et al., 1977), 0.13 pmol T3 $min^{-1}mg^{-1}$ (Kaplan and Utiger, 1978) and 0.07 pmol T3 $min^{-1}mg^{-1}$ (Chopra et al., 1979) for a rat liver microsomal preparation; and of 1.2 pmol T3 $min^{-1} mg^{-1}$ (Leonard and Rosenberg, 1980b) for a rat kidney microsomal

preparation. An earlier report of the velocity for a rat liver micro-

somal preparation of approximately 0.02 pmol T3 $min^{-1}mg^{-1}$ (Hesch et al.,

1975) was low due to absence of added cofactor (thiol compound such as

mercaptoethanol or dithio-threitol during incubation). Another report of

a maximum velocity of 63 pmol T3 $min^{-1}mg^{-1}$ (Visser et al., 1979a) for a

rat liver microsomal preparation appeared to be extremely high when com-

pared to previously reported values and those observed in the present

study, especially since the reported purification of the microsomal pre-

paration was only 2- to 3-fold greater than the crude rat liver homo-

genate which had a reported Vmax of 2.9 pmol T3 $min^{-1}mg^{-1}$ (Visser et

al., 1976b).

Among other factors, such as differences in pH, ionic strength, and

T4 substrate concentrations, a major factor contributing to differences

in reported Km and Vmax values for the 5'-deiodinase was differences in

cofactor and cofactor concentration. In the present study, for example,

an increase in dithiothreitol concentration in the incubation buffer from

3 to 20 mmol/L DTT resulted in an increase in Km of from 1.03 to 4.35

μmol/L T4 and an increase in Vmax of from 65.8 to 285 pmol T3 $min^{-1}mg^{-1}$ for the Fraction III 5'-deiodinase. Though not as apparent in the

crude rat liver homogenate (Chopra, 1977b), this phenomenon has also been

observed for rat liver microsomal preparations (Visser, 1979) and rat

kidney microsomal preparations (Leonard and Rosenberg, 1978). Despite

this fact, all previous determinations of kinetic constants for the 5'-

deiodinase have been calculated for less than optimal cofactor concen-

trations. In the present study, replots of the kinetic data (Figure 27)

generated from apparent Km and Vmax values obtained at different concen-

trations of dithiothreitol were utilized to determine the true kinetic parameters (Dixon and Webb, 1979a).

The parallel increase in Km and Vmax with increasing dithiothreitol concentration generated a family of parallel lines when double reciprocal plots of velocity versus T4 concentration for the 5'-deiodinase were plotted with respect to varying concentrations of dithiothreitol (Figure 26). This type of kinetic behavior is characteristic of that observed for two-step transfer reactions (Cleland, 1963), and indicated that the conversion of T4 to T3 might occur by a ping-pong type mechanism:
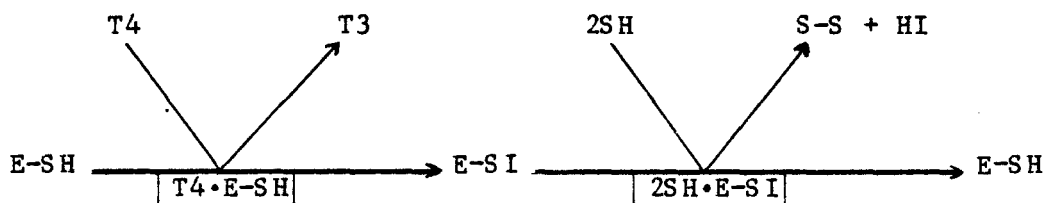
According to the ping-pong reaction mechanism, the reductive deiodination of T4 would occur in 2 consecutive steps. In the first step, the $C_{5'}$-iodine of the outer ring of T4 would be removed generating the first product, T3. In the second step, the oxidized enzyme intermediate (E-SI), which was proposed to be a sulphenyl iodide (Visser et al., 1979b; Leonard and Rosenberg, 1980b) would be reduced back to its original active form (E-SH) and HI would be released as the second product as 2 equivalents of the thiol group-containing cofactor were oxidized. The occurrence of parallel lines in the double reciprocal plot of velocity versus substrate concentration for different concentrations of cofactor has also been observed for the conversion of T4 to T3 in rat kidney microsomal preparations (Leonard and Rosenberg, 1978) and for the conversion of rT3 to 3,3-'T2 in rat liver microsomal preparations (Visser, 1979b), however, the present data provides the first evidence that the conversion of T4 to

T3 by the rat liver T4 5'-deiodinase occured by the same mechanism.

The fact that the kinetic data resulted in parallel lines in the double reciprocal plot suggests, but does not prove that the 5'-deiodinase obeyed a true two-step transfer mechanism. Compulsory-order equilibrium systems also generate parallel lines in double reciprocal plots when the product of the substrate dissociation constant (K_S) for substrate A (T4) and the Michaelis-Menten constant (Km) for substrate B (DTT) are very small in comparison to the product of the concentrations of substrates a and b ($K_S^A \cdot Km^B/a \cdot b$) (Dixon and Webb, 1979a). One way to distinguish between the two systems is to use a competitive inhibitor of substrate A. If the lines in the double reciprocal plot appeared parallel because the value of K_S^A was very low, carrying out the kinetic determinations in the presence of a fixed amount of competitive inhibitor can be used to increase the apparent K_S^A and thereby result in a convergence of the plotted lines for varying concentrations of substrate B (Dixon and Webb, 1979b). Figure 28 shows the double reciprocal plot for the 5'-deiodinase as determined in the presence of 75 μmol/L sodium salicylate which was found in the present study to be a competitive inhibitor of the Fraction III 5'-deiodinase with respect to both thyroxine and dithiothreitol. The generation of parallel lines in Figure 28, therefore, gave added support to the hypothesis of ping-pong kinetics.

In the present study, substrate inhibition by T4 was observed at low detergent concentrations when the concentration of T4 was increased above 20 μmol/L T4 in the presence of 20 mmol/L DTT (Figure 25), and when DTT concentrations were increased above 20 mmol/L DTT in the presence of

ferent isoenzymes of the T4 5'-deiodinase were present in the rat liver microsomal preparation. In the present study, no evidence was observed for the presence of multiple isoenzymes of the T4 to T3 converting enzyme during the determination of molecular weight and isoelectric point, or during the determination of catalytic parameters for the purified rat liver 5'-deiodinase.

The fact that crude rat liver and kidney preparations are active in the 5'-deiodination of both T4 and rT3, and the observation that the conversion of T4 to T3 and the conversion of rT3 to 3,3' T2 are similar in many respects has led to an ongoing debate over whether or not the same enzyme is responsible for the reductive deiodination of the 2 idothyronines. Evidence supportive of a one enzyme hypothesis is based on the observations that the subcellular distribution of both activities are similar (Maciel et al., 1979; Leonard and Rosenberg, 1980a), that both activities are affected in a similar manner by a variety of inhibitors including propylthiouracil, iodoacetic acid and ipodate (Oragrafin) (Chopra, 1978; Eisenstein et al., 1980), and that rT3 and T4 inhibit the deiodination of each other in a dose dependent manner (Kaplan and Utiger, 1978; Visser et al., 1979a). Evidence supportive of the existence of separate enzymes for the deiodination of T4 and rT3 is based primarily on temporal differences in the early abrupt rise in serum T3 concentrations and the delayed decline in serum rT3 concentrations which occur postnatally in the neonate (Chopra et al., 1975a). These differences have been interpreted to indicate that the conversion of T4 to T3 and the clearance of rT3 are carried out by different pathways (Chopra, 1978; Maciel et al., 1979). Additionally, differences in optimum pH observed for the

conversion of T4 to T3 (pH 6.5) and for the conversion of rT3 to 3,3'-T2 (pH 8.0) (Visser et al., 1979a) are also suggestive of the existence of separate enzymes. In the present study, enzyme assays utilizing ^{125}I-labeled substrates demonstrated that the crude solubilized 5'-deiodinase (Fraction D) was active in the deiodination of both T4 and rT3. Therefore, both activities were present in the membrane bound fraction of the rat liver homogenate and both activities solubilized in an active form in the presence of Nonidet P-40. The purified 5'-deiodinase preparation (Fraction III), on the other hand, demonstrated a minimal capacity to deiodinate rT3. On an equal volume basis, Fraction III was approximately 20 times less active than the crude preparation in the deiodination of rT3 despite the fact that it was at least twice as active as Fraction D in the conversion of T4 to T3. Therefore, the rT3 deiodinase activity of Fraction D was either removed or inactivated during purification. The fact that the addition of Fraction D to Fraction III did not result in reactivation of the Fraction III rT3 deiodinase activity suggested that a loss of cofactor during purification was not responsible for the low rT3 deiodinase activity of the Fraction III preparation. The failure of the rT3 5'-deiodinase activity to co-purify with the T4 5'-deiodinase plus the fact that the deiodination of T4 and rT3 occured optimally at pH 6.5 and pH 7.5 or higher, respectively is, therefore, strongly suggestive that different enzymes might be involved in the deiodination of T4 and rT3.

SUMMARY

Recent reports of reciprocal changes in serum 3,3,5'-triiodo-
thyronine (T3) and 3,3',5'-triiodothyronine (rT3) levels during starva-
tion, stress and febrile illness have stimulated interest in what appears
to be a physiologically regulated, control mechanism whereby the body can
fine tune the biological effects of the thyroid hormones. A previously
unpurified, membrane-bound iodothyronine 5'-deiodinase, which activates
thyroxine (T4) by converting it to the more physiologically active hor-
mone T3, is thought to be primarily responsible for control of this path-
way.

The purpose of this study was to solubilize and partially purify
the iodothyronine 5'-deiodinase from rat liver membranes and to charac-
terize the purified enzyme in terms of substrate specificity, catalytic
parameters, molecular weight, and isoelectric point. The mechanism of
action of the enzyme was also examined.

Results indicated that 0.7 % Nonidet P-40 was effective in solubil-
izing up to 60 % of the 5'-deiodinase activity from rat liver membranes.
After solubilization, the 5'-deiodinase was purified 580-fold. The puri-
fied enzyme eluted in a single peak during analytical gel filtration, and
a single peak of 5'-deiodinase activity was also observed during isoelec-
tric focusing. The active complex had an apparent molecular weight of
250,000 and an apparent isoelectric point of 7.4.

Kinetic assays demonstrated that the purified 5'-deiodinase was
optimally active at 37 °C and pH 6.5, and that there was an absolute re-

quirement for the presence of a thiol group-containing cofactor. The most potent cofactor, dithiothreitol (DDT), was used in this work. The Km value for T4 was dependent on the concentration of cofactor and parallel lines in the Lineweaver-Burk plot were generated when kinetic data for the conversion of T4 to T3 were plotted for varying concentrations of DTT. This data as well as other observed catalytic properties of the purified enzyme were consistent with the hypothesis of a ping-pong mechanism of action for the rat liver T4 5'-deoidinase. At saturating substrate and cofactor conditions, the Km values for T4 and DDT were determined to be 5.0 μmol/L and 11.4 mmol/L, respectively, and the Vmax for the purified enzyme was 320 pmol T3 $min^{-1}mg$ $protein^{-1}$. Reverse T3 was an apparent non-competitive inhibitor with respect to both T4 and DTT. Cholografin, sodium salicylate and propranolol were dose dependent, competitive inhibitors with respect to both T4 and DTT. Propylthiouracil inhibited the reaction un-competitively with respect to T4 and competitively with respect to DTT.

Despite a high binding affinity for rT3 ($K_I = 9.9 \times 10^{-8}$ mol/L), the purified 5'-deiodinase did not actively deiodinate rT3.

These data suggest the existence of a single specific thryoxine 5'-deiodinase in rat liver membranes that catalyzes the conversion of T4 to T3, and that can be solubilized and purified in the presence of Nonidet P-40 as a dispersing agent. The fact that the purified enzyme did not catalyze the 5'-deiodination of rT3 is interesting because it suggests the possible existence of a specific reverse T3 5'-deiodinase, and therefore, that the peripheral deiodination scheme for the iodothyronines may be more complex than orignally anticipated.

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