Hepatic 5′Deiodinase Activity of Japanese Quail

Using Reverse-T$_3$ as Substrate: Assay Validation,

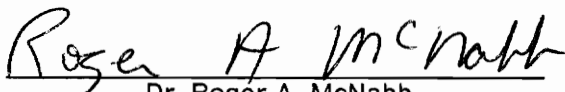**Characterization, and Developmental Studies**

by

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(ABSTRACT)

Using reverse-triiodothyronine, rT$_3$, as substrate, an *in vitro* 5'deiodinase (5'D) assay was validated for adult Japanese quail, by defining conditions under which activity is proportional to enzyme (protein) concentration and is linear with incubation time. Activity was measured as the release of I^{125} from labeled rT$_3$. Using validated assay conditions we found the following 5'D characteristics: maximal activity from 10-50 mM dithiothreitol (cofactor), an apparent K$_m$ of 0.52 μM rT3, pH optimum of 7.6-8.5, complete inhibition by 1 mM propylthiouracil and by 1 mM iopanoic acid, and substrate affinities of rT$_3$ > T$_4$ > T$_3$. Based on these characteristics, the quail hepatic 5'D activity is like the Type I 5'D activity found in mammalian liver and kidney, and embryonic chicken liver. To determine how previous unvalidated assays, that used high tissue concentrations and relatively low substrate (T$_4$) concentrations, influenced 5'D studies we reevaluated 5'D development using our assay validated for each developmental stage. We found extreme quantitative differences in the activities measured and in the proportional relationships among stages; and only limited qualitative similarity existed in the pattern of 5'D development when compared to unvalidated T$_4$ assays. These data show good correspondence between whole liver 5'D activity per unit body weight and plasma T$_3$/T$_4$ ratios for the developmental stages sampled.

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Chapter I

LITERATURE REVIEW

OVERVIEW OF THYROID PHYSIOLOGY

Thyroid hormones play crucial roles in the regulation of metabolism, thermogenesis, growth and development, and reproduction. Regulation of thyroid function is necessary for maintenance of homeostasis and for response to changes in the external environment. Thyroid hormones are produced within the follicles of the thyroid gland and are stored in the colloid of the follicles as the glycoprotein, thyroglobulin. Thyroglobulin is engulfed by the follicular epithelial cells, and the thyroid hormones, released by hydrolysis of the thyroglobulin molecule, are secreted into the circulation (see reviews by Gorbman *et al.*, 1983; DeGroot *et al.*, 1984; Norris, 1985; Taurog, 1986; Norman and Litwack, 1987; Tepperman and Tepperman, 1987).

The secretion (production and release) of thyroid hormones from the thyroid gland is regulated by negative feedback involving the hypothalamic-pituitary-thyroid axis (HPT axis). Thyroid hormone secretion is stimulated by thyrotrophin (TSH, thyroid stimulating hormone), a glycoprotein trophic hormone produced and released by the anterior pituitary gland. Se-

cretion of TSH is, in turn, inhibited by the negative feedback of T_3 at the pituitary gland, and is stimulated by thyrotrophin-releasing hormone (TRH) and inhibited by somatostatin from the hypothalamus (Norris, 1985; Utiger, 1987). Thus, the regulation of overall thyroid economy is based on integration of information about both the external environment and the internal state of the organism (see reviews by Gorbman et al., 1983; DeGroot et al., 1984; Norris, 1985; Taurog, 1986; Norman and Litwack, 1987; Tepperman and Tepperman, 1987).

The major secretory product of the thyroid gland, and the major circulating form of thyroid hormone, is thyroxine or tetraiodothyronine (T_4). In mammals T_4 is considered a prohormone, and triiodothyronine (T_3) is considered to be the physiologically active thyroid hormone (see review by Oppenheimer, et al., 1987). This is supported by the presence of nuclear T_3 receptors, but not T_4 receptors, in tissues that respond to thyroid hormones. Such receptors are present in all vertebrate classes (Oppenheimer et al., 1987; Bellabarba et al., 1988; Galton, 1983, 1988; Weirich, 1988). T_3 is produced within the thyroid gland, either by direct synthesis from iodotyrosine precursors or by the deiodination of T_4 during hormone release. The hydrophobic nature of thyroid hormones requires that they must be transported in the circulation bound to serum binding proteins (see review by Robbins and Bartalena, 1986). Approximately 99% of the circulating thyroid hormones are bound to such transport proteins; the remaining free or unbound fraction is in equilibrium with the bound fraction. It is this free fraction that is available to enter cells of target tissues (see review by Ekins, 1986).

Most of the T_3 in the body is produced by the extrathyroidal deiodination of T_3 (see reviews by Chopra et al., 1978; Engler and Burger, 1984; Hennemann, 1986). This process, a key step in the regulation of thyroid hormone effects, involves the enzymatic removal of an iodide from the 5′ position of the T_4 molecule by 5′deiodinase (5′D). T_3 produced by deiodination either remains within the cell, and may bind to nuclear receptors to initiate a response, or returns to the circulation from which it can enter other tissues (see reviews of mammalian studies by Kaplan, 1986; Leonard and Visser, 1986; Hennemann, 1986; and reviews of comparative 5′D studies by Decuypere and Kuhn, 1988; Galton, 1988; McNabb, 1988; McNabb and Freeman, 1990).

Thyroid hormones are disposed of by sequential deiodination to successively less iodinated forms or by other processes. In order of importance these processes are (1) conjugation with glucuronide or sulfate, (2) formation of acetic acid analogs via deamination and decarboxylation and (3) other oxidative process such as cleavage of the ether linkage (Burger, 1986; DiStefano, 1988).

The Role of Extrathyroidal Deiodination in Thyroid Hormone Dynamics in Mammals

The extrathyroidal deiodination of T_4 is a key step in the regulation of plasma T_3 concentrations, and in the distribution and availability of T_3 among tissues throughout the body. In euthyroid man, approximately 80% of the T_3 produced daily is derived from extrathyroidal deiodination of T_4 (Engler and Burger, 1984). Liver and kidney are the most important sources of T_3 for supply to the circulation, T_3 produced by deiodination in some other tissues (e.g., brain, pituitary gland, brown adipose tissue) largely remains within the tissue of production. For example, in the euthyroid rat 50% of the T_3 bound to nuclear receptors within the anterior pituitary gland is derived from T_4 5'D within the tissue (the remainder is derived from plasma T_3), while in the liver and kidney only 28% and 14% of T_3 bound to nuclear receptors was derived from local T_4 5'D, respectively (Silva *et al.*, 1978; Crantz *et al.*, 1982).

Types of Deiodination Reactions

The deiodination of T_4 is complex. T_4 can undergo either 5'-deiodination of the outer ring to produce T_3, or 5-deiodination of the inner ring resulting in the formation of 3,3',5'-triiodothyronine (reverse-T_3, rT_3). Either of these products can undergo subsequent deiodinations resulting in less iodinated thyronines. 5'deiodination of T_4 is an activating pathway because it produces the physiologically active hormone, T_3. 5-deiodination of T_4, resulting in rT_3, is a deactivating pathway because rT_3 has no known physiologic function. Reverse-T_3 can also undergo 5'-deiodination producing 3,3'-diiodothyronine (3,3'-T_2). Removal of an iodine from T_3 also results in formation of a T_2 and results in disposal of the active

thyroid hormone. Subsequent deiodinations also occur, but these pathways have received little attention (see review Hesch and Koehrle, 1986).

Deiodinase Pathways

There are three functionally-defined types or pathways of 5'D, each with distinct biochemical and physiological properties and tissue distributions (see Table 1 and reviews by Leonard and Visser, 1986; Hesch and Koehrle, 1986; and Kaplan, 1986). The activity of these pathways results in the production of most of the circulating T_3, provides for local regulation of T3 concentration in some tissues, and also plays a role in the disposal of "excess" T_4 and T_3. The regulation of 5'D activity is tissue-specific, and is important in adjustments to some physiological states (e.g., hypothyroidism and hyperthyroidism, food availability and composition, and developmental stages) and to some environmental conditions (Kaplan, 1986).

The three forms or isozymes of deiodinase activity are classified on their experimentally determined functional properties. The primary criteria for pathway classification are: (1) Michaelis constant (K_m), (2) sensitivity to 1 mM propylthiouracil (PTU), (3) order of substrate affinities for iodothyronine substrates (T_4, rT_3, and T_3), (4) the "key" reaction catalyzed (5'D, 5D or both), (5) tissue distribution and (6) responses to certain physiological alterations (Kaplan, 1986; Leonard and Visser, 1986). See Table 1 for a characterization of each deiodinase pathway.

Type I Deiodinase: Type I deiodinase activity is found primarily in liver, kidney and the thyroid gland. Type I deiodinase can catalyze both the activating (5'D) and the deactivating (5D) pathways of T_4, metabolism producing T_3 and rT_3, respectively. To date it has not been possible to separate the 5'D and 5D activities (see review by Leonard and Visser, 1986) and it is assumed that a single enzyme catalyzes both (Hesch and Koehrle, 1986).

Type I deiodinase has a higher affinity (lower Michaelis constant) for rT_3 than for T_4. *In vitro*, and probably *in vivo*, any rT_3 present is rapidly deiodinated to $3,3'-T_2$. While rT_3 has no known physiologic action it may play a role in regulating intracellular production of T_3 through

competition with T_4 for the deiodinase enzyme. Because rT_3 is a substrate for 5'D, has a lower K_m, and undergoes less complex metabolism than T_4, it is now considered the substrate of choice for *in vitro* studies of 5'D (Leonard and Rosenberg, 1980; and see discussion below).

The 5'D contains sulfhydryl group(s) that are essential for enzyme activity. Sulfhydryl groups are oxidized during the deiodination reaction, and then must be reduced to return the enzyme to its native state. Subcellular fractionation experiments indicate that the enzyme is located in the microsomal fraction, and reveal the presence of a heat-labile "cofactor" present in the cytosolic fraction (Balsam *et al.*, 1979; Kaplan *et al.*, 1979; Maciel *et al.*, 1979). The physiologic cofactor has yet to be identified, however, a variety of thiol cofactors have been tested *in vitro* (Goswami and Rosenberg, 1984, 1988). Glutathione (GSH) is the most abundant non-protein sulfhydryl compound in the cell, but large changes in cellular GSH concentrations have little effect on 5'D activity (Sato and Robins, 1981; Sato, *et al.*, 1983). Increases in oxidized glutathione (GSSG) correlate with decreases in 5'D activity (Sato *et al.*, 1983). It has been proposed that the ratio of reduced to oxidized glutathione may regulate 5'D activity (see review by Leonard and Visser, 1986). The artificial cofactor dithiothreitol (DTT) is most commonly used for *in vitro* experiments.

The Type I deiodination reaction probably proceeds through an ordered ping-pong reaction mechanism (Leonard and Rosenberg, 1978, 1980). It has been proposed that the iodide of the substrate reacts with the essential sulfhydryl in the active site of the enzyme resulting in the formation of T_3 (if T_4 is the substrate) and an enzyme-sulfenyl-iodide intermediate. In the second step the thiol cofactor reacts with the sulfenyl-iodide intermediate, restoring the sulfhydryl of the enzyme to the reduced state and releasing iodide (Hesch and Koehrle, 1986; Leonard and Visser, 1986).

Propylthiouracil (PTU) inhibits Type I deiodinase activity *in vivo* and *in vitro*. PTU is known to form mixed disulfides with proteins. It is believed that PTU reacts covalently with the enzyme-sulfenyl-iodide intermediate to form a stable inactive enzyme-PTU disulfide (Visser and van Overmeeren-Kaptein, 1981; Hesch and Koehrle, 1986). Early in the development of this field, inhibition by 1 mM PTU was used as the sole criterion to distinguish Type I from Type

II activity (the latter is PTU-insensitive, see below). It is now recognized that sensitivity to PTU in itself is not sufficient to distinguish deiodinase pathways (see the work of Silva et $al.$, 1987). Iopanoic acid is a competitive inhibitor of 5'D and completely abolishes activity of all pathways at 1 mM.

Most of the T_3 produced by Type I 5'D activity in the liver and kidney is supplied to the circulating T_3 pool. The level of Type I 5'D activity is controlled primarily by T_4 (substrate) availability. Therefore, hepatic and renal 5'D activities are decreased in hypothyroidism and increased in hyperthyroidism (see section below).

Type II Deiodinase: Type II deiodinase is a true 5'D found in the central nervous system, pituitary gland and brown adipose tissue. This activity can 5'deiodinate T_4 to T_3, and rT_3 to T_2; however, it differs from the Type I pathway in that it prefers T_4 as substrate, i.e., has a lower K_m for T_4 than for rT_3. It also differs from the Type I deiodinase in having a K_m in the nanomolar range (1-2 nM) and a much higher thiol requirement (20 mM DTT is typically used for in $vitro$ assays as opposed to 1-5 mM for Type I deiodinase assays) (Silva and Larsen, 1986; Leonard and Visser, 1986).

Type II 5'D is relatively or completely insensitive to 1 mM PTU. At low DTT concentrations in $vitro$, it is partially inhibited by PTU, but as the DTT concentration is increased the inhibition by PTU is diminished and disappears in the presence of 20 mM DTT. Presumably the DTT has access to the disulfide linkage between the PTU and the sulfenyl-iodide-enzyme intermediate and is able to restore the enzyme to its native form. Alternatively, Visser et $al.$, (1983) provide evidence that enzyme-sulfhydryl groups do not participate in 5'deiodination by the PTU-insensitive pathway.

Kinetic experiments suggest that the Type II deiodinase functions through a sequential reaction mechanism (Hesch and Koehrle, 1986; Leonard and Visser, 1986). where both the substrate and the cofactor bind to the active site of the enzyme prior to catalysis (Dixon and Webb, 1979).

In $vivo$ kinetic experiments involving the injection of I^{125}-T_4 and I^{131}-T_3 have been used to trace the sources of plasma and intracellular thyroid hormones. These studies indicate that

greater than 50% of the T_3 produced by Type II deiodinase activity of euthyroid rat brain and pituitary gland remains within the cell and accounts for a large fraction of the nuclear bound T_3 (Silva *et al.*, 1978; Crantz *et al.*, 1982). The physiological significance of this retention of T_3 within the cells where it is formed is discussed in sections on central nervous system and pituitary gland, and sections on hypothyroidism and hyperthyroidism.

The primary signal for regulation of Type II 5'D activity is plasma T_4 concentration (McCann *et al.*, 1984; Silva and Leonard, 1985). Type II deiodinase activity is inversely related to the concentration of plasma T_4. In hypothyroidism, Type II deiodinase activity increases in brain and thereby maintains a euthyroid status within the tissue, ensuring normal function. In the pituitary, Type II 5'D activity plays an important role in the regulation of TSH production and release (see sections central nervous system and pituitary gland, and on hypothyroidism and hyperthyroidism; see review by Hesch and Koehrle, 1986).

Type III Deiodinase: Type III deiodinase is an inner ring or 5D found in brain and placenta. This pathway displays a sequential kinetic pattern, prefers T_3 over T_4, and has a K_m of approximately 40 nM. It is stimulated by thiol cofactors. Brain Type III activity is insensitive to PTU, but placental Type III activity is inhibited by PTU if the cofactor concentration is less than 1 mM. Type III deiodinase activity increases with plasma T_4 concentrations and plays a role in deactivating T_4, and in the disposal of T_3. (Huang, *et al.*, 1986; Kaplan, 1986; Leonard and Visser, 1986).

Tissue Distribution

Each of the deiodinase pathways has a characteristic distribution among the tissues of the body. The compartmentalization of deiodinase activity allows for local regulation such that the requirements of individual tissues, as well as those of the organism as a whole, can be met.

Liver and Kidney: Liver and kidney have been the tissues most widely employed in studies of deiodination. The first characterizations of 5'D were carried out using liver and kidney tissue, and this activity is what is now considered to be Type I (high K_m) deiodinase

(initially only a single pathway was recognized). Today, despite some suggestions that a low K_m deiodinase activity is present in these tissues (Goswami and Rosenberg, 1984, 1985), it is generally accepted that liver and kidney contain only Type I deiodinase activity (Silva *et al.*, 1987).

Liver and kidney generally are believed to be the most important sources of circulating T_3. Compared with other tissues, most of the T_3 produced by liver and kidney is supplied to the circulation. Silva *et al.*, (1978) demonstrated that only 28% and 14% of the T_3 bound to nuclear receptors in liver and kidney, respectively, was derived from local deiodination, while greater than half of the T_3 bound to nuclear receptors in the cerebellum (50-60%) and cerebral cortex (70-80%) was derived locally (Crantz *et al.*, 1982). Further, when the high deiodinase activity and large size are considered, it is clear that liver and kidney have a tremendous potential for producing plasma T_3.

Thyroid gland: The thyroid gland contains deiodinase activity that is considered to be Type I deiodinase. Laurberg and Boye (1982) compared the biochemical characteristics of the deiodinase activity in preparations of tissue from the thyroid with those of liver from the same dogs and found the two activities to be very similar. After T_4 is released from engulfed thyroglobulin by lysosomal proteases, 5'D can convert some of the T_4 to T_3 prior to secretion into the circulation (see reviews by Laurberg, 1984; Hesch and Koehrle, 1986).

Thyroidal 5'D activity is increased in response to TSH stimulation. This is particularly important in hypothyroidism, where increased thyroidal deiodinase activity, stimulated by elevated TSH levels, increases the T_3/T_4 ratio of the gland effluent. This helps to maintain plasma T_3 availability, despite great reductions in hepatic and renal deiodinase activity (see section on hypothyroidism and hyperthyroidism). Thyroidal 5'D is the only known tissue deiodinase that responds to TSH stimulation (Erickson *et al.*, 1982, Wu, 1983; Wu *et al.*, 1985).

Central Nervous System and Pituitary Gland: Several studies in the early 1980's were suggestive of the presence of a second pathway of T_4 deiodination with different physiological and biochemical characteristics than that of liver and kidney. For example, despite the fact that the Type I pathway is completely inhibited by PTU, PTU administration to rats *in vivo* fails

to decrease intrapituitary T_3 concentration (Silva and Larsen, 1978; Cheron et al., 1979; Kaplan, 1980). Similarly, PTU administration in vivo at levels that completely inhibit hepatic and renal deiodinase activity in vitro fails to completely suppress serum T_3 concentration in T_4-replaced thyroidectomized rats (Oppenheimer, et al., 1972; Bernal and Escobar del Rey, 1974; Frumess and Larsen, 1975; Larsen and Frumess, 1977). These observations led to the discovery and characterization of the PTU-insensitive or Type II 5'D activity (Silva et al.,1982; Visser et al., 1982, 1983a, 1983b). Type II 5'D is the predominant pathway in the central nervous system and the pituitary gland (Larsen et al., 1981; Silva and Larsen, 1982).

In the euthyroid state Type II 5'D produces T_3 that remains within the tissue, and is the source of approximately 50% of the T_3 bound to nuclear receptors. The remaining 50% is derived from the plasma T_3 pool. Thus, the pituitary gland is responsive to plasma levels of T_3 directly, and T_4 indirectly via intrapituitary deiodination to T_3. The occupancy of nuclear T_3 receptors within the pituitary gland is inversely correlated with TSH secretion. Therefore, pituitary 5'D activity, in concert with circulating T_3, plays an important role in the negative feedback control of TSH production (see reviews by Larsen et al., 1981; Silva and Larsen, 1986).

Brain and pituitary gland respond rapidly to alterations in thyroid hormones by increasing or decreasing Type II 5'D activity (Leonard et al., 1981; Silva and Leonard, 1985; Silva and Larsen, 1986). For example, in hypothyroidism Type II 5'D activity increases rapidly, thereby increasing T_3 production within the brain and maintaining normal brain function despite the hypothyroid state of the remainder of the body (See section on hypothyroidism and hyperthyroidism).

Type III activity is present in the cerebral cortex, and is three-fold higher in fetal rats than in adult rats. This pathway helps to regulate intracellular thyroid hormone concentrations by converting T_4 to rT_3, thereby preventing T_3 production (Cooper et al., 1983). It also removes T_3 from the system by converting it to T_2 (Kaplan and Yaskowski, 1980; Huang, et al., 1986; Kaplan, 1986; Kodding et al.,1986).

Brown Adipose Tissue: Brown adipose tissue (BAT) contains Type II deiodinase activity (Leonard *et al.*, 1983; Goswami and Rosenberg, 1986; Silva and Larsen, 1985, 1986). BAT Type II activity plays a central role in the thermogenic response of BAT during cold-exposure (Biancho and Silva, 1986; see section below), and in hypothyroidism it can supply significant amounts of T_3 to the circulation, a characteristic unlike that of Type II activity in other tissues.

Placenta: Type III activity 5-deiodinates T_4 to rT_3 and T_3 to T_2 and, therefore, represents a deactivating pathway that protects the fetus from high levels of T_3 and T_4 (El-Zaheri *et al.*, 1981). Thyroid hormones play crucial role(s) in growth and development, thus it is necessary that their levels be carefully regulated. There also have been suggestions that high T_3 concentrations can be toxic to embryos (mammals: Huang, *et al.*, 1986; birds: Borges *et al.*, 1980).

Other Tissues: Deiodinase activity is known to exist in other tissues, however, other than a survey of several rat tissues these have received little attention (Chopra, 1977; McCann, *et al.*, 1984). Skeletal muscle may prove to be a fruitful area of study. Based on *in vivo* kinetic studies and mathematical models it has been proposed that skeletal muscle may be a more important source of circulating T_3 than has been appreciated previously (DiStefano, 1986, 1988; Jang and DiStefano, 1986). Muscle appears to have low deiodinase activity per unit tissue (Chopra, 1977), and there is a slow exchange of thyroid hormones between the plasma and the intracellular compartments (due to the structure of capillaries within muscle tissue). However, the large mass of muscle may compensate for the low activity and slow exchange (DiStefano, 1986).

Responsiveness to Physiological Alterations

The activity of the deiodination pathway(s) in each tissue is regulated independently of other tissues. This allows the organism to achieve a coordinated response of its tissues and organs to changes in internal state or in the external environment.

Hypothyroidism and Hyperthyroidism: In hypothyroidism and hyperthyroidism the activity and distribution of tissue deiodinases changes to maintain a euthyroid state in "critical" tissues, despite altered thyroid hormone availability. In response to reduced plasma T_4 avail-

ability in hypothyroidism, brain Type II deiodinase activity increases resulting in increased production of T_3 which remains largely within the tissue (van Doorn et al., 1986); brain Type III 5D activity decreases, resulting in reduced clearance of T_3. The net result of these alterations is the maintenance of a euthyroid state within the brain, independent of the hypothyroid condition prevailing in the remainder of the body. This protects normal brain function in the adult, as well as normal brain development and maturation in young animals, from the hypothyroid condition (Leonard and Visser, 1986; Silva and Larsen, 1986).

The deiodinase activities in the pituitary gland respond to circulating hormones in a similar manner. The significance, in this case, is that monitoring of the status of both thyroid hormones is achieved, with the availability of plasma T_4 (the prohormone) as the primary information. For example, if plasma T_3 is decreased, but plasma T_4 is normal (suggesting adequate hormone production by the thyroid gland), intrapituitary deiodination of T_4 to T_3 will be sufficient to prevent the "interpretation" that reduced plasma T_3 alone constitutes a hypothyroid condition (Larsen et al., 1981).

In hypothyroidism, the source of plasma T_3 is shifted away from liver and kidney (van Doorn et al., 1986), to increased T_3 production by BAT, and increased T_3/T_4 ratios in the venous effluent from the thyroid gland. High plasma TSH concentrations are the trigger for the increased thyroid Type I 5'D activity, which increases the proportion of T3 secreted from the thyroid (Erickson et al., 1982; Wu, 1983; Wu et al., 1985). Very low T_4 levels lead to a complete cessation of hepatic and renal Type I deiodinase activities, and to a three- to four-fold increase in BAT Type II deiodinase activity (Silva and Larsen, 1985, 1986). In contrast to other tissues with Type II deiodinase activity, T_3 produced by BAT Type II makes a significant contribution to circulating T_3 concentrations.

The response of tissue deiodinases to hyperthyroidism is generally the opposite of that observed during hypothyroidism. Type I and Type III deiodinases are increased (with the exception of Type I deiodinase activity in the thyroid gland), resulting in an increased turnover and clearance of thyroid hormones. Type II deiodinase activities are decreased, and thereby

help to maintain euthyroid T_3 concentrations within the brain and pituitary gland, and to suppress production of plasma T_3 by BAT.

Dietary Influences: Following the onset of fasting there is a rapid decline in hepatic 5'D activity, resulting in a decrease in plasma T_3 (Balsam *et al.*, 1978). Plasma T_4 concentrations remain unchanged, or increase slightly, and plasma rT_3 may be increased. The reduction in plasma T_3 results in a decreased metabolic rate, which serves to conserve available energy stores (Danforth, 1986). The mechanism behind the decreased 5'D activity is not known, but clearly this is not mediated by changes in plasma T_4 concentrations as is usually the case for Type I deiodinase activity. The decrease in activity is at least partly related to limitation of endogenous cofactor during fasting (Balsam *et al.*, 1981; Danforth, 1986; Kaplan, 1986).

Refeeding following fasting rapidly restores hepatic 5'D activity and plasma T_3 concentrations to control levels. These changes allow the organism to return to pre-fasting metabolic rates. Long-term overfeeding in humans results in an increase in hepatic 5'D activity. The resultant increase in plasma T_3 concentration increases the utilization of available energy, thereby limiting storage of excess energy. This is accomplished by a T_3-induced increase in metabolic rate (diet-induced thermogenesis) as well as increases in specific enzymatic pathways. For example, activity of α-glycerophosphate dehydrogenase is increased with increased plasma T_3 concentration (Danforth, 1986; Kaplan, 1986).

Temperature: BAT plays a central role in thermoregulation in mammals by non-shivering thermogenesis in response to cold-exposure during the neonatal period and in postnatal life, and in diet-induced thermogenesis. Sympathetic stimulation of BAT results in increased Type II deiodinase activity, although the mechanism by which this occurs is unknown. Much of the T_3 produced by this pathway binds to nuclear receptors within BAT and induces the production of thermogenin, the protein that uncouples oxidative phosphorylation (Biancho and Silva, 1987a). Upon sympathetic stimulation there is an increase in the oxidation of fatty acids and uncoupling of respiration, resulting in high heat production without ATP generation (Biancho and Silva, 1987a, 1987b, 1987c).

Development

The key event in peripheral thyroid hormone metabolism during development is a shift from the predominantly deactivating (5D) to activating (5'D) pathways in liver. The physiological significance of this pattern is that it allows development of a fully functional HPT axis without exposing the organism to concentrations of T_3 that might be high enough to disrupt the synchrony of developmental events, or overstimulate metabolic rate. In mammals, the fetus is largely protected from exposure to maternal T_4 and T_3 by the Type III deiodinase activity in the placenta (El-Zaheri et al., 1981; Huang et al., 1986; Kaplan, 1986).

The timing of the shift from deactivating to activating pathways of thyroid hormone metabolism varies with species and depends on the mode of development (precocial versus altricial). Studies of several species of mammals and birds indicate that the HPT axis matures prior to birth or hatching in precocial species, and that there is a perinatal peak in 5'D activity. In altricial species the HPT axis matures later, during early postnatal life, and activity increases gradually during the postnatal period.

The perinatal peak in hepatic 5'D activity in precocials appears to be triggered by increases in glucocorticoids (Wu et al., 1978) and by high substrate (T_4) concentration resulting from TSH stimulation of the thyroid. Presumably, increases in endogenous cofactor during the perinatal period play a role, because embryonic tissues have been shown to have reduced cofactor (Harris et al., 1978; Sato et al., 1982, 1983) as well as less 5'D enzyme than adult tissues (Kaplan and Yaskowski, 1981, 1982; Wu et al., 1986b). Following the perinatal peak there is a gradual reduction in deiodinase activity to adult levels. Deiodinase pathways have been measured in several tissues during development (McCann et al., 1984), however, liver and brain are the only tissues for which adequate assay validations have been performed.

Development of the central nervous system clearly is dependent on thyroid hormones. Severe hypothyroidism in the neonatal rat results in decreases in many thyroid-hormone-dependent maturational changes (Silva and Larsen, 1982). During less severe hypothyroidism deiodinase pathways within the brain respond in the manner described earlier (see section on hypothyroidism and hyperthyroidism) to protect brain T_3 concentrations and thyroid-

hormone-dependent events (Kaplan and Yaskowski, 1981, 1982; McCann *et al.*, 1984; Silva and Matthews, 1984).

Avian Deiodinase Studies

Studies of avian deiodinases have focused on whole animal deiodination or on *in vitro* hepatic deiodination. Based on these studies avian deiodinases appear to be very similar to mammalian enzymes in both biochemical characteristics and in responses to physiological and environmental alterations. In adult birds hepatic 5'deiodinase activity changes with dietary status, in response to thyroid hormone availability, in response to changes in ambient temperature, and over the course of development.

During development in precocial birds (chicken and quail), there is the shift from pre-dominantly inactivating pathways to activating pathways as described earlier (Borges *et al.*, 1980, 1981; Galton and Hiebert, 1987; the present study). In Japanese quail, hepatic 5'deiodinase activity has been measured as early as day 12 of a 16.5 day incubation (the present study), and in chickens as early as day 15 of a 21 day incubation (Galton and Hiebert, 1987). There appears to be a very good correlation between hepatic 5'deiodinase activity and plasma T_3 and T_3/T_4 ratios during development (the present study). This suggests that in birds, as in mammals, hepatic 5'D (and possibly renal 5'D) may be the major source of plasma T_3.

In the one altricial avian species that has been studied (ring doves), there was very low 5'D activity prior to hatch, and hepatic 5'D activity increased in the early post-hatch period (McNabb and Cheng, 1985; Rieman and McNabb, in press). However, deactivating pathways of thyroid hormone metabolism have not been investigated in any altricial birds.

No published study of avian deiodination, except that of Galton and Hiebert (1987), Rieman and McNabb (in press) and the present study, demonstrate that the assay conditions used to measure 5'D activity are valid, i.e. measure initial reaction velocities. Use of initial velocity assay conditions is essential if measurements of enzyme activity and comparisons of "treatments" are to be valid (Dixon and Webb, 1979; Engel, 1981). Due to the complex metabolism of T_4 (Chiraseveenuprapund *et al.*, 1978; Leonard and Rosenberg, 1978; Balsam

and Ingbar, 1978; Galton and Hiebert, 1987), it is extremely difficult to obtain initial velocity conditions using T_4 as substrate. Therefore, rT_3, because of its simple metabolism is now considered to be the substrate of choice for *in vitro* deiodinase assays (Leonard and Rosenberg, 1980; Galton and Hiebert, 1987). Freeman and McNabb (in press) compared measurements of hepatic 5'D activity during development in Japanese quail, using the typical unvalidated T_4 assay conditions with those made using validated assay conditions and rT_3 as substrate. The results were strikingly different quantitatively, but showed some qualitative similarity in the characterization data. The results of avian deiodinase studies to date probably generally reflect the correct pattern of tissue deiodinase activity and the responses to physiologic and environmental alterations. However, care must be taken in interpreting the results until further investigations using validated assay conditions are performed. (For a more detailed discussion see chapter 2.)

Table 1. Summary of the Characteristics of Mammalian Deiodinase Pathways[*]

	TYPE I	TYPE II	TYPE III
Reaction Catalyzed	5'D and 5D	5'D	5D
Substrate preference	rT3 > T4 > T3	T4 > rT3	T3 > T4
Km for T4	～1 uM	～1 nM	～40 nM
Effect of Thiols	stimulatory	stimulatory	stimulatory
Reaction Mechanism	ping-pong	sequential	sequential
Effect of Propylthiouracil	inhibitory	no effect	no effect[a]
Tissue Distribution	liver kidney thyroid	CNS BAT and pituitary	CNS and placenta
Response to Hypothyroidism	decrease[b]	increase	decrease
Predominate Destination of T3 Produced	plasma	intracellular[c]	———

[a] some inhibition in placenta.

[b] Thyroidal Type I 5'D increases in response to TSH.

[c] in hypothyroidism and cold-exposure significant amounts of T3 produced by BAT Type II 5'D enters the circulation.

[*] modified from that of Kaplan (1986).

Chapter II

HEPATIC 5'-DEIODINASE ACTIVITY OF JAPANESE QUAIL USING REVERSE-T3 AS SUBSTRATE: ASSAY VALIDATION AND CHARACTERIZATION.

INTRODUCTION

There have been many studies of 5'D activity in birds, and most have used liver tissue from chickens and employed T_4 as substrate. The methods used in these avian T_4 5'D studies were adopted from several mammalian studies (Harris *et al.*, 1978; Kaplan and Utiger, 1978; El-Zaheri *et al.*, 1980) that were performed at a time when this field was relatively new. Neither these mammalian studies nor the subsequent avian studies that established the techniques in the avian literature (Decuypere *et al.*, 1983; Scanes *et al.*, 1983; Rudas, 1986; Hughes and McNabb, 1986) performed assay validation experiments. Such validations are necessary to demonstrate that the experimental conditions employed measure initial velocity,

as is necessary for making valid treatment comparisons with enzymatic assays (Dixon and Webb, 1979; Engel, 1981). Galton and Hiebert (1987) defined and used initial velocity conditions in their study of reverse-T_3 5'D in embryonic chicken liver, but validation studies have not been included in any published study of adult tissues from any avian species (see review by McNabb and Freeman, 1990).

Initial velocity conditions are those in which enzyme activity increases proportionally with increasing enzyme concentration, and increases linearly (and proportionally) with increasing incubation time. The methods used to measure T_4 5'D in avian tissues (citations above) have employed high homogenate concentrations, relatively low substrate concentrations and relatively long incubation times. We have tested these conditions in three avian species (quail, chickens and doves) and demonstrated that they do not measure initial velocities (unpublished studies, our laboratory). These results suggest that factors such as substrate limitation, product inhibition or enzyme inactivation are limiting the reaction. In addition, studies with mammalian liver and kidney, using T_4 as substrate, have shown that initial velocity conditions often cannot be achieved because of the presence of multiple reactions (Chiraseveenuprapund et al., 1978; Leonard and Rosenberg, 1978; Balsam and Ingbar, 1978). Both 5'D and 5D reactions are catalyzed by a single enzyme (Type I) in these tissues (Leonard and Visser, 1986) and both these reactions can occur with T_4 as substrate (Leonard and Rosenberg, 1980). Deiodination of products prior to measurement can cause further problems. Thus, if any of the multiple reactions described above are occurring, measurement of only one product will underestimate the amount of enzyme present.

To avoid such problems most recent studies of 5'D in mammalian liver and kidney have used rT_3 as substrate. Galton and Hiebert (1987) also used rT_3 as substrate in their assays because they found the deiodination of T_4 in embryonic chicken liver to be complex.

There are no published studies of 5'D activity in adult avian tissues that have demonstrated the conditions necessary for measuring enzyme activity at initial velocity. Our objectives in this study were (1) to validate a rT_3 5'D assay for use with adult quail liver by

defining initial velocity conditions, and (2) to use those conditions to characterize the rT_3 5'D activity and (3) to reevaluate the development of 5'D in quail liver.

MATERIALS AND METHODS

Tissue Preparation

Tissue pools were prepared from the livers of at least five adult female Japanese quail (*Coturnix japonica*) for the validation and characterization studies. Eight embryos or chicks per age were sampled for the developmental studies. Quail were reared and maintained as previously described (McNabb *et al.*, 1986). The birds were sacrificed by decapitation and the livers were removed rapidly and placed in ice-cold MOPS buffer (50 mM morpholinopropane sulfonic acid with 1 mM EDTA at pH 7.4). Livers were blotted dry, weighed to the nearest 0.01 g, minced and homogenized in 3 vol (wt/vol) of ice-cold MOPS buffer in a glass/teflon coaxial Potter-Elvehjem tissue grinder held in ice-water. Three types of tissue preparations were used in the first set of studies.

Homogenate: The 25% crude homogenate (wt/vol) described immediately above was centrifuged at 1000g at 4 C for 10 min. Aliquots of this supernatant, hereafter referred to as homogenate, were frozen rapidly in liquid nitrogen and stored at -10 C. For each experiment an aliquot was thawed rapidly (37 C) and diluted to the desired concentration with MOPS buffer. When handled and stored in this manner the tissue retained all enzymatic activity for at least 6 weeks.

Postmitochondrial fraction (PMF): For most experiments a PMF of the 25% crude homogenate (wt/vol) was prepared in MOPS buffer by a procedure similar to that of Galton and Hiebert (1988). The homogenate was centrifuged at 12,100g at 4 C for 2 minutes, and the supernatant was held on ice. The pellet was washed by resuspension, in a volume of MOPS buffer equal to the volume of supernatant and recentrifuged as before. The combined supernatant and wash yields a 12.5% PMF (original tissue wt/vol) which was snap-frozen and stored as described above.

Microsomes: Liver microsomes were prepared by the method of Aronson and Touster (1974), which was used by Leonard and Rosenberg (1978) for preparation of crude subcellular

fractions for use in T_4 5'D assays. The microsomal pellet was washed with assay buffer and resuspended to yield a final volume equivalent to the volume of the initial 25% crude homogenate (wt/vol). Aliquots of the microsomal suspension were snap-frozen and stored as described above.

Assay of 5'D Activity

The standard assay, with an incubation volume of 80 μl, consisted of the following: 25 μl tissue homogenate or fraction, 20 μl dithiothreitol solution (DTT; Calbiochem-Behring, San Diego, CA), 10 μl of MOPS buffer, 20 μl of unlabeled rT_3 solution (Calbiochem-Behring), and 5 μl of the I^{125}-labeled rT_3 solution (approximately 100,000 cpm; SA, 750-1250 μCi/μg; New England Nuclear Corp., Boston, MA). The radioactive rT_3 was labeled randomly in the 3'- or 5'-position of the outer ring. All reagents were prepared in MOPS buffer. Unless otherwise indicated final concentrations of 4.0 μM rT_3, 20 mM DTT, and 10 min incubations were used in the experiments below. Labeled rT_3 was diluted with MOPS buffer to the desired concentration immediately before use. The rT_3 concentration of 4 μM is approximately 10x the apparent K_m of the 5'D. With such a high substrate concentration saturation of the enzyme will be approached, resulting in near maximal activity and independence from substrate concentration (Cornish-Bowden, '76a).

All reaction components were pipetted into 6x50 mm culture tubes (held on ice). After vortexing the tubes, the rack of tubes was placed in a shaking waterbath (37 C, 90 strokes per minute). After incubation, the reaction was terminated and the I^{125} released was measured. Blanks consisted of tissue-free incubations, or samples (with tissue) in which the reaction was terminated immediately after the addition of substrate. Protein concentrations were determined for the tissue preparation used in each experiment.

Measurement of I^{125} released: When 5'D activity was measured as the rate of radioiodine released from labeled rT_3, the reaction was terminated by the addition of 20 μl 4% BSA, followed immediately with 150 μl ice-cold 20% trichloroacetic acid. The tubes were held in ice-water for 15 minutes then total cpm were determined. After centrifugation at 4000g at -10

C for 30 min, I^{125} present in the supernatant was measured by the method of Leonard and Rosenberg (1980). Briefly, 170 μl of the supernatant was applied to Bio-Rad Dowex AG 50W-X8(H+) ion exchange columns (Bio-Rad Laboratories) that had been washed with 10 ml 1.74 M acetic acid. Radioiodine was eluted with four 2 ml aliquots of 1.74 M acetic acid and the I^{125} in the acetic acid fractions was counted.

The use of the Dowex columns was validated according to the procedure of Leonard and Rosenberg (1980). Greater than 98% of the iodide was recovered in the first two fractions with not more than 0.56% in the latter two fractions combined. Precision was 0.94% and 2.20% ($\pm$ 2SE as % of the mean; n=10) in two experiments.

Iodide release was calculated from the sum of the iodide recovered in the four fractions from each sample, corrected back to the original sample volume and expressed as a percent of the total cpm. This value was corrected for percent iodide in the blank and then multiplied by two (to account for random labeling of rT_3 in the 3'- and 5'- positions).

Protein determinations: The Bradford Protein Assay (Biorad Laboratories, Richmond, CA), modified to accommodate the small sample volumes available, was used. The standard curve was 0 to 1.4 μg BSA/μl in MOPS buffer, sample/standard volume was 40 μl, dye reagent volume was 2.0 ml. Other details of the assay were carried out according to the instructions of the manufacturer. All standards and samples were assayed in duplicate. Precision of the assay was 0.5% ($\pm$ 2SE as % of the mean; n=10).

Assay Validation

To establish initial velocity assay conditions we determined the range over which activity is proportional to tissue (enzyme) concentration using homogenate, PMF, and microsomes. Then we determined the relationship between activity and incubation time for two concentrations of PMF (equivalent to 0.05 and 0.10 mg original tissue/tube). We worked with reactions that consumed <10% of the substrate.

To verify the presence of only 5' deiodination of rT_3, we separated the products by column chromatography. Equivalency of iodide and $3,3'T_2$ produced demonstrates the lack

of subsequent degradation of the T_2. For these studies the reaction was terminated by the addition of 20 μl of MOPS buffer containing unlabeled T_4, rT_3 and PTU (1 mg/ml each) followed immediately by 100 μl ice-cold absolute ethanol. After 16 to 24 hr at -10 C, total cpm were determined for each tube. Tubes were centrifuged at 4000g at -10 C for 30 min, the ethanol extract was removed and diluted to 500 μl with phosphate buffer (pH 6.5, 1 N NaCl), and pipetted onto a 1.9 x 15 cm Sephadex (G-25 superfine) column. This was followed by 10 ml 0.01 N NaH_2PO_4, and then 0.01 N NaOH from a reservoir, and 160 fractions of 20 drops each were collected and counted. Peaks of radioactivity were identified by comparing their position of elution with that of known labeled iodothyronines and iodide. Because labeled 3,3'-T_2 was not available, this peak was identified by its position in relation to other labeled iodothyronines (Butt, 1976). The amount of each compound in a sample or blank was calculated as a percentage of the cpm under a peak expressed as a fraction of total cpm recovered.

Characterization of rT3 5'D Activity

Initial velocity conditions defined by the validation experiments were used (4.0 μM rT_3, 20 mM DTT, PMF equivalent to 0.10 mg original tissue/tube and a 10 min incubation). Changes from these conditions for each part of the characterization are described below. Additional hormones or inhibitors were dissolved in 10 μl MOPS buffer. (These replaced the 10 μl MOPS buffer in the standard assay volumes described above).

The cofactor range tested was 0-50 mM DTT. For the kinetic experiments, substrate concentrations were 0.05-4.0 μM rT_3, (approximately 0.1 to 10 times the apparent K_m) and PMF equivalent to 0.10 mg original tissue/tube was used with 4 μM rT_3, and PMF equivalent to 0.05 mg original tissue/tube was used for other substrate concentrations. Incubation times were varied with substrate concentration (5 min for 0.05-0.2 μM rT_3, 7.5 min for 0.4 μM rT_3 and 15 min for 0.8-4.0 μM rT_3) to ensure that enough product was accumulated for accurate measurements, and so that less than 10% of the substrate was consumed. Propylthiouracil (PTU) sensitivity was tested at (1) 1.0 mM PTU with 1, 5, 10, and 20 mM DTT for 10 min and (2) 1.0 mM PTU with 20 mM DTT, from 0 to 25 min. Iopanoic acid (IOP) sensitivity was tested at

1.0 mM. Substrate preference was tested by coincubation with (1) 0-18 μM unlabeled T_4 and (2) 0-18 μM unlabeled T_3.

The effect of pH on 5'D activity was measured from pH 5.5-9.0 in 0.5 pH steps. Livers from 4 adult quail were pooled, minced and 0.5 g of tissue was homogenized in 3 vol of buffer at each pH (pH 5.5-9.0, in 0.5 pH steps). The buffer consisted of 100 mM sodium acetate, 100 mM Tris, and 100 mM potassium phosphate (Kaplan *et al.*, 1983). Hepatic PMF was prepared at each pH as described above. DTT and rT_3 solutions were made as 9-fold concentrates in MOPS buffer (pH 7.4) and diluted with buffer of the appropriate pH. Labeled rT_3 was diluted in MOPS buffer as described above. The final reaction mixture consisted of 11.2 μl MOPS buffer and 68.8 μl buffer of the appropriate pH. During the experiment, pH was measured in parallel incubations lacking only labeled hormone. Reaction mixture pH was stable with time.

Developmental Studies

Four developmental ages were sampled (12, 14 and 16 day embryos, and 1 day chicks). Three of these (14 and 16 day embryos and 1 day chicks) corresponded to ages sampled by Hughes and McNabb (1986) in an earlier study. Hatching animals were not included, because adequate resolution of the data during this period of rapid and extreme changes requires sampling at several "stages," every few hours. At the time of sampling, aliquots of 12.5% PMF from individuals, as well as, pooled samples for each age were snap-frozen and stored. Assay validation studies were performed on the pooled tissue for each age prior to determining 5'D activity for each individual.

Statistical Analyses

Regression analyses were made using an unweighted least squares procedure. For the kinetic studies, the K_m, V_{max}, and their standard errors were calculated by the statistical method of Cornish-Bowden (1976b). Where appropriate, data were analyzed by one-way analysis of variance and Duncan's Multiple Range test. Values of $P < 0.05$ were accepted as indicative of statistically significant differences.

RESULTS

Validation studies using reverse-T_3

Proportionality of activity (pmol I- released) with enzyme concentration was demonstrated using homogenate (up to 0.288 mg homogenate protein/ml of reaction mixture; data not shown), PMF (up to 0.325 mg PMF protein/ml) and microsomes (up to 0.675 mg microsomal protein/ml; data not shown) with 20 mM DTT (Fig. 1a). When expressed as specific activity (pmol I- released/mg protein), activity was constant over the proportional range (Fig. 1b). PMF was used in all experiments reported below.

Linearity of activity (pmol I- released) with incubation time for at least 25 minutes (Fig. 2) was demonstrated using two protein concentrations within the proportional range (0.175 and 0.085 mg PMF protein/ml in the reaction mixture; representing approximately 0.10 and 0.05 mg original tissue/tube, respectively). The specific activity (pmol I- released/min-mg protein) was identical for the two dilutions, further verifying the proportionality between activity and protein under these conditions. The presence of only 5'-deiodination of rT_3 (without additional degradation of products) was verified by demonstrating the equivalency of I- and $3,3'T_2$ produced for duplicate tubes from the linearity experiment (data not shown). The activity measured by this method was not significantly different from that based on iodide separated on Dowex columns.

Characterization studies

Using the initial velocity conditions defined above, the rT_3 5'D activity was characterized. In the absence of DTT, thiol-independent activity was apparent at 1 min of incubation (earliest measurement) with no further thiol-independent activity after this time. This activity was equivalent to that measured in the presence of 20 mM DTT at 1 minute. Therefore in studies of the effect of DTT, this activity was subtracted from that measured at each DTT concentration. Activity increased rapidly up to 10 mM DTT and, thereafter, was essentially

unchanged up to 50 mM DTT, the highest concentration used. Three replicate experiments gave the same results. Lineweaver-Burke analysis of these data indicated an apparent Michaelis constant (K_b) for DTT of 2.04 mM at a fixed rT_3 concentration of 4.0 μM.

The apparent K_m and V_{max} values of rT_3 5'D were 0.52 μM rT_3 and 355 pmol I-released/min-mg PMF protein, respectively, when measured in the presence of 20 mM DTT (Fig. 3). Enzyme activity with rT_3 was highest, and did not differ significantly between pH 7.6 and 8.5. Reaction mixture pH was stable during incubation. rT_3 5'D activity was completely inhibited by 1.0 mM PTU in the presence of 1, 5, 10 or 20 mM DTT. Inhibition was complete by 5 min (earliest measurement) of a 25 min incubation. Enzyme activity was completely inhibited by 1.0 mM IOP, a competitive inhibitor of 5'deiodination. Thyroxine inhibited rT_3 5'D activity in a dose-dependent manner. There was 50% inhibition at 13 μM T_4. Coincubation with T_3 (up to 18 μM in the reaction mixture) had no effect on rT_3 5'D activity (data not shown) although T_3 is deiodinated when neither rT_3 nor T_4 are present in the reaction mixture (unpublished data, our laboratory).

Developmental Studies

Validation studies using pooled tissue from each developmental stage indicated that the assay conditions established for adult tissues also measured initial velocities in developing tissues. Activity was proportional to enzyme concentration up to at least 0.2 mg tissue/tube (as PMF protein) and linear with incubation time for at least 20 min.

Specific activity of 5'D increased significantly (~3 fold) in embryos between day 12 and day 16 when the embryos had pipped into the air cell (Fig. 4a, solid bars). The specific activity in 1-day chicks was lower than that in 16 day embryos. The developmental pattern was the same whether specific activity was expressed per unit of PMF protein, or per unit of liver tissue.

Fig. 4b shows the potential liver T_3 supply to the circulation as whole liver 5'D activity in relation to changing body size during development (solid bars). These values were obtained by multiplying specific activity (pmol I-/min-mg liver) by total liver weight and dividing by body

weight. The two methods of expressing 5'D activity are compared with the pattern of plasma T_3 concentrations and the T_3/T_4 ratios measured previously in our laboratory (McNabb and Hughes, 1983) in Fig. 4 a and b. Within the present study, whole liver 5'D activity in relation to body weight (solid bars, Fig. 4b) showed better correspondence with plasma T_3/T_4 ratios (solid line, Fig. 4a and b) than did specific activity of 5'D (Fig. 4a). Plasma T_3 concentrations (dashed line, Fig. 4b) did not fit well with either presentation of 5'D activity.
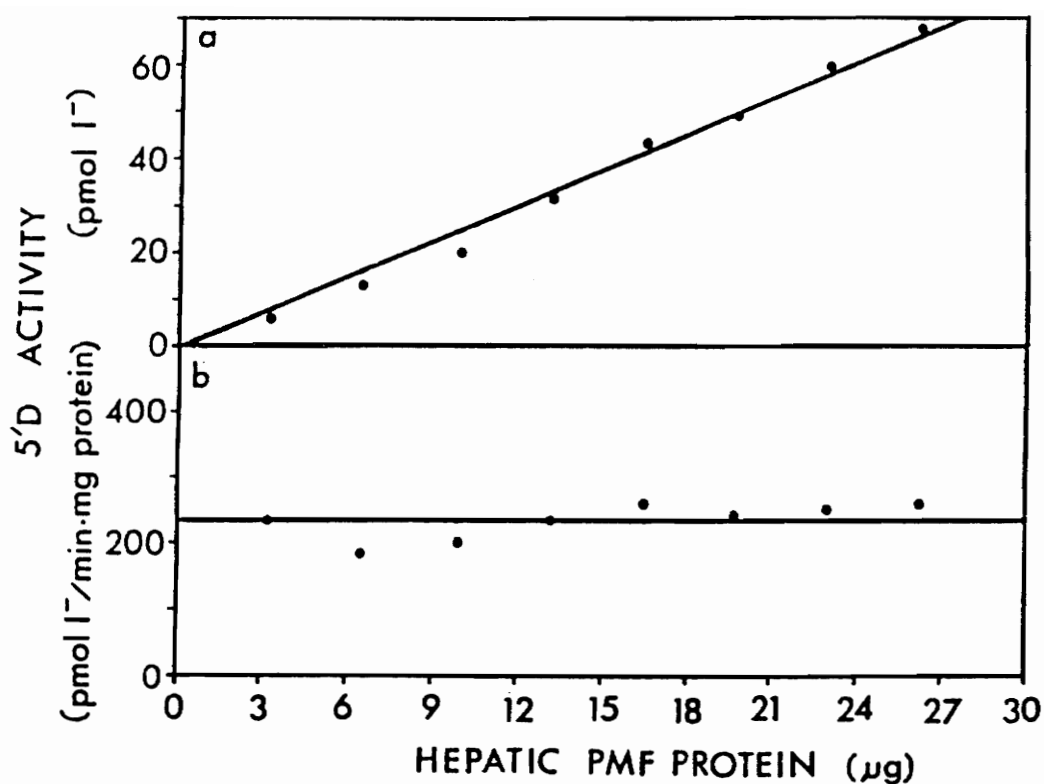
Figure 1. Proportionality of hepatic rT_3 5′D activity with enzyme (postmitochondrial fraction protein) concentration: Liver was pooled from five adult Japanese quail. Assay conditions: 4 μM rT_3, 20 mM dithiothreitol, 10 min incubation. Data reported are the means of duplicate determinations. (a) pmol iodide generated per 10 min, (b) pmol iodide generated per min-mg protein.
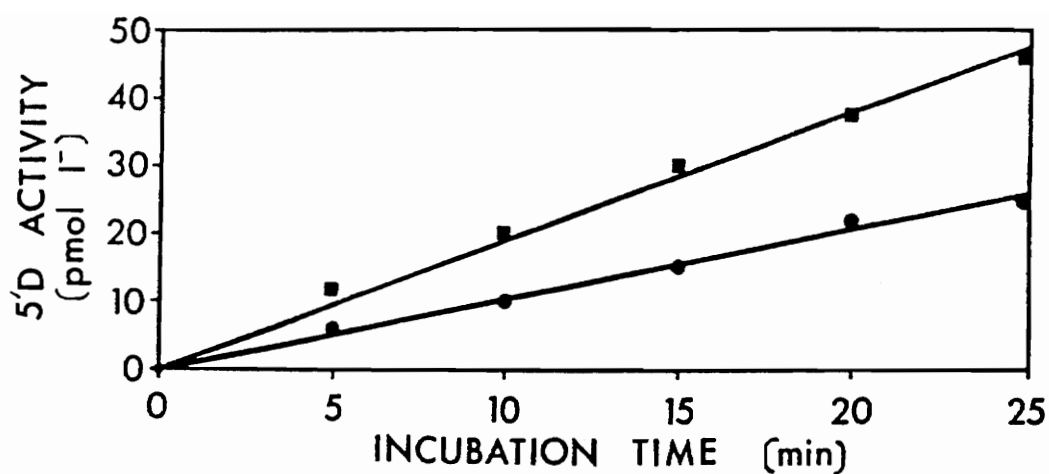
Figure 2. Linearity of hepatic rT$_3$ 5'D activity with Incubation time: Postmitochondrial fraction was prepared from liver from five adult Japanese quail. Two concentrations of PMF protein, .085 (-•-) and .175 (-■-) mg PMF protein/ml in the reaction mixture (equivalent to .05 and .10 mg original tissue/tube), were incubated with 4 μM rT$_3$ and 20 mM dithiothreitol. Data reported are the means of duplicate determinations.
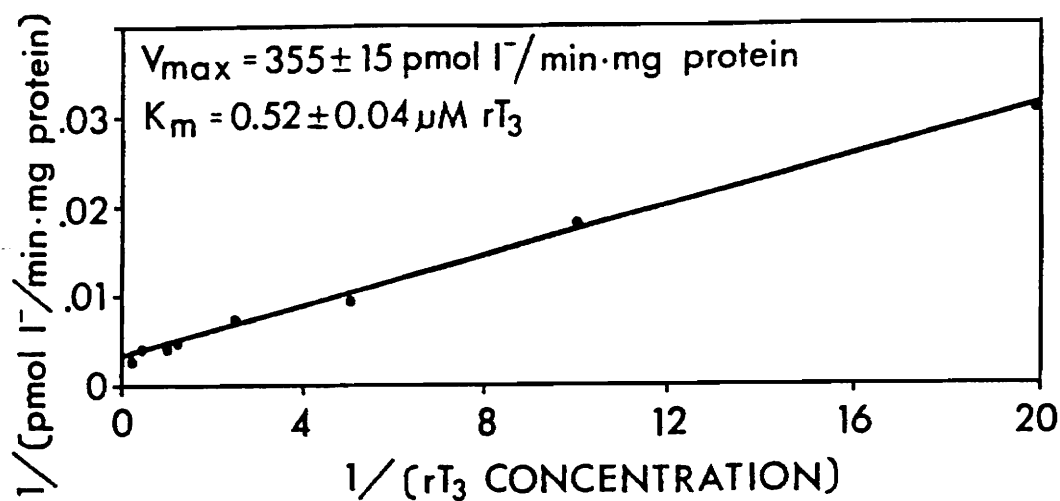
Figure 3. Lineweaver-Burke plot of initial velocity kinetics of hepatic rT₃ 5'D activity.: Postmitochondrial fraction was prepared from liver pooled from five adult Japanese quail. Dithiothreitol concentration was fixed at 20 mM. Data points are the means of triplicate determinations. K_m, V_{max}, and their standard errors were calculated by the statistical method of Cornish-Bowden (1976b).
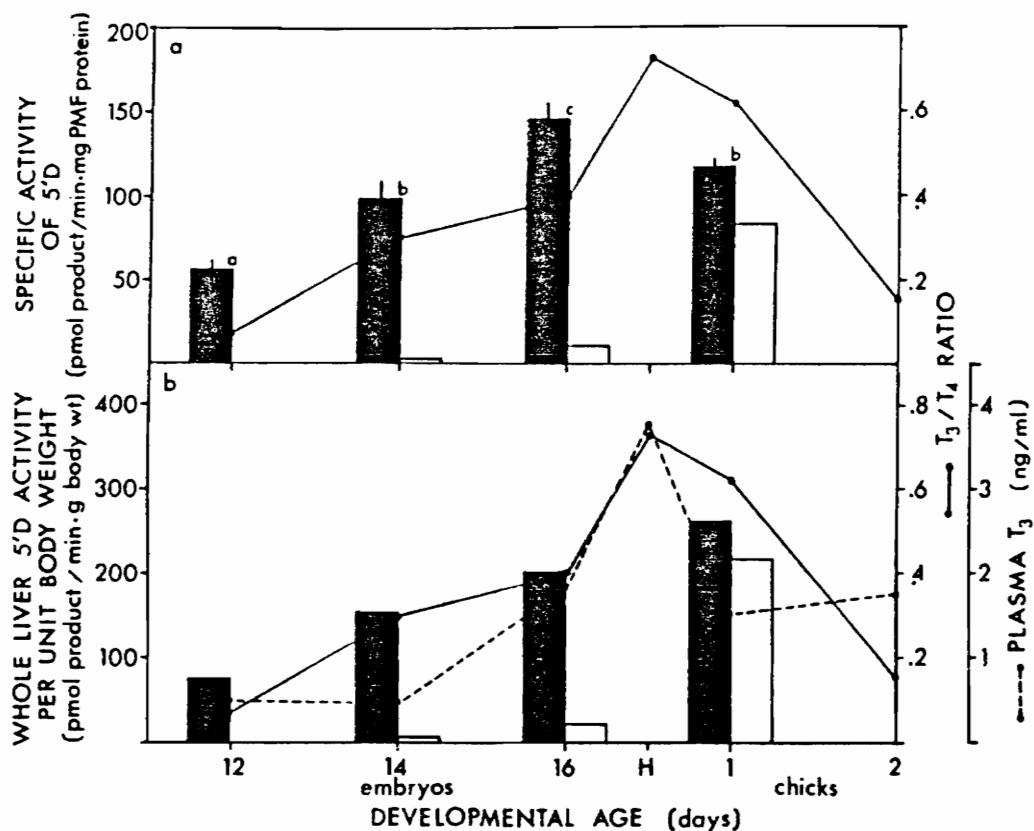
Figure 4. Hepatic rT_3 5'D activity at several stages of development in Japanese quail: (a) Specific activity of 5'D, (b) whole liver 5'D per unit body weight. Embryonic ages are given as days of the 16.5 day incubation period, H = hatching, chicks were 1 day of age. Data are presented as means ± SE. Stages with the same letter designation do not differ statistically. Dark bars, data from the present study; open bars, data from Hughes and McNabb (1986) multiplied by 5000. Solid line, plasma T_3/T_4 ratios; dashed line, plasma T_3 concentrations (data from McNabb and Hughes, 1983).

DISCUSSION

Most studies of avian 5'D activity have used T_4 as substrate but have not validated their assays, so the results must be questioned (see review by McNabb and Freeman, 1990). Attempts to define valid T_4 assay conditions, i.e. those that measure initial enzyme velocity, have not been successful for liver tissue from embryonic chickens (Galton and Hiebert, 1987) or adult chickens, quail or doves (unpublished studies in our laboratory). This may be due to the presence of multiple deiodination reactions in the assay; Galton and Hiebert (1987) have shown that both 5D and 5'D are present, and that when T_4 is deiodinated it appears that the T_3 formed is degraded rapidly to $3,3'T_2$ and I-.

Assay Validation

In the present study we describe validation studies that demonstrate initial velocity conditions and characterize the 5'D activity using rT_3 as substrate. Our studies show that proportionality of rT_3 5'D activity with enzyme concentration can be achieved using three different kinds of tissue preparation - homogenate, PMF, and microsomes. The range of enzyme (tissue) concentrations over which we demonstrated proportionality were similar to those used by Galton and Hiebert (1987) in their microsomal preparations from embryonic chicken liver, and by Leonard and Rosenberg (1980) in microsomal preparations from rat kidney. The validation data (proportionality with enzyme concentration and linearity with time) presented in this study illustrate how different sets of assay conditions could be selected for making measurements under initial velocity conditions. Measurement of all the labeled products demonstrated that only 5'D activity was occurring, based on stoichiometric equivalency of $3,3'T_2$ and I- produced.

Characterization Studies

Characterization studies of the 5'deiodinase in adult quail liver, with rT_3 as substrate, showed stimulation by thiol cofactor (with a high cofactor requirement compared to that when T_4 is used as substrate), "preference" for $rT_3 > T_4 > T_3$ as substrate, a relatively high K_m (μmolar range), and complete inhibition by 1 mM PTU or IOP. These characteristics are similar to those of mammalian liver and kidney deiodinase (see review by Leonard and Visser, 1986).

Our kinetic studies indicate an apparent K_m of 0.52 μM with rT_3 as substrate for quail hepatic 5'D, a value similar to many others in studies using rT_3 and liver or kidney tissue of rats (0.46 μM, Leonard and Rosenberg, 1980; 0.50 μM and 0.53 μM, Goswami and Rosenberg, 1984 and 1985, respectively), but not chickens (0.118 μM, Rudas, 1986; 0.141-0.145 μM, Galton and Hiebert, 1987).

Our studies of PTU inhibition demonstrate that, like rT_3 5'D of rat liver and kidney, quail liver rT_3 5'D is completely inhibited by 1.0 mM PTU. The conditions of our assay and this inhibition by PTU suggest that we were measuring only a Type I activity in the present study. This is in contrast to our previous report of partial PTU-sensitivity of quail liver T_4 5'D (McNabb *et al.*, 1986). The contrast between these studies remains unresolved, but could have been due to our earlier study using a high enzyme:substrate ratio (see discussion by Silva *et al.* 1987). Additional studies using nanomolar concentrations of T_4 as substrate and other conditions suited to Type II activity would be needed to rule out the possibility of Type II activity.

Other studies also have reported that avian hepatic 5'D has similar characteristics to that in mammals (embryonic chickens, Galton and Hiebert, 1987; adult chickens, Rudas, 1986; adult quail, McNabb *et al.*, 1986). Thus, despite some of these studies using inappropriate assay conditions with consequent low quantitative estimates of 5'D activity, there is some qualitative similarity in the characteristics reported. We speculate that this is because characterization studies use a single pooled tissue preparation to measure, for e.g., the effect of different pH conditions. If a pooled tissue preparation is more concentrated than the range

that gives proportionality, the activity values will be inappropriately low, but the pH optimum could be the same as in determinations at initial velocity conditions.

Developmental Studies

Our studies of four developmental ages indicated that specific activity of 5'D increased almost three-fold from 12 to 16 days of embryonic life, then decreased in 1 day chicks to levels comparable to those in 14 day embryos (Fig. 4a). However, specific activity may not be the best expression of 5'D to relate to the developing organism, because *in vivo* T_3 production will depend not only on specific activity of the enzyme (which reflects enzyme content per unit of liver) but also on the size of the organs producing T_3 and the availability of T_4 as substrate. Therefore, to relate the 5'D data to the whole organism during development, we expressed the potential for 5'D activity in relation to liver and body growth (whole liver 5'D per unit body weight; Fig. 4b). When the data are expressed in this way, it is apparent that 5'D activity increases through the embryonic stages and continues to increase in 1 day chicks (Fig. 4b). This expression of 5'D activity has merit because T_3 produced by the liver and released to the circulation will be distributed in a blood volume that is proportional to body weight, and thus should be related to the plasma hormone picture.

When we compared the developmental patterns of plasma hormones with both expressions of 5'D activity, we found the best correspondence between T_3/T_4 ratios and whole liver 5'D per unit body weight (solid line and dark bars, Fig. 4b). This result seems reasonable because the T_3/T_4 ratio accounts for T_4, which is the substrate for the 5'D activity, as well as relating the total liver T_3 production to its circulatory distribution within the whole organism.

We compared the 5'D developmental data in the present paper with that of Hughes and McNabb (1986; open bars, Fig. 4a,b) to evaluate how the previous picture of physiological "treatments" (developmental stages) was altered by measurements at initial velocity conditions. The results suggest very limited qualitative similarity in the general pattern of 5'D development, and extreme differences in the quantitative values obtained by the two studies. The limited similarity is in both studies showing the highest 5'D activity in the perinatal period,

and lower activities in mid to late embryos and in chicks. The differences in the quantitative results from the two methods are extreme (note 5000 fold multiplication of the Hughes and McNabb data in Fig. 4) and there is no consistent quantitative relationship between the values from the two studies for specific developmental stages. It seems unlikely that the differences in specificity of the enzyme for rT_3 vs. T_4 as substrate could account for more than a small fraction of the quantitative differences. Although we have not been able to establish a validated T_4 assay for avian tissues, using T_4 and assay conditions that approach initial velocity (using much lower enzyme:substrate ratios than in previous studies) yield specific activity measurements at least a thousand times higher (unpublished studies in our laboratory) than those reported by the avian studies that have used T_4 as substrate.

Our measurements of 5'D activity in quail embryos are similar to the V_{max} values reported by Galton and Hiebert (1987) for chicken embryos at equivalent stages. Both studies also show a 3X increase in 5'D activity in equivalent periods during late incubation (days 15-21 of the 21 day chicken incubation period, and days 12-16 of the 16.5 day quail incubation period).

Another example of how treatment comparisons, made using inappropriate assay conditions, can result in erroneous information, is given by the study of Wu et al. (1986). These authors were able to find a very limited range of conditions under which they could measure initial velocity with T_4 as substrate in fetal rat liver (Wu et al., 1985). Using these conditions, they were able to show that the lower 5'D activity in the fetus was due to less enzyme in fetal than in adult liver. Previously, unvalidated assays with high homogenate and low substrate concentrations led to differential limitation of activity in the assay of fetal and adult tissues and the erroneous conclusion that the low fetal activity was caused primarily by cofactor deficiency (Chopra, 1978).

Summary

This study has developed valid assay conditions for studying 5'D activity in an adult avian tissue (quail liver), using labeled rT_3 as substrate and measuring activity as I^{125} released. Such validated assays can be used for comparing treatments because differences in the 5'D activity measured should reflect only differences in the enzyme content of the tissues from different treatments.

We have demonstrated that the conditions used in many previous studies of avian 5'D (high homogenate concentrations, relatively low substrate concentrations, T_4 as substrate) do not measure initial velocities, and thus underestimate enzyme activity. Neither we, nor Galton and Hiebert (1987), were able to establish valid assay condition using T_4 as substrate.

In other studies where inappropriate assay conditions were used, the characteristics of the enzyme from a single tissue pool have given some qualitatively similar information, although the activities measured were quantitatively far less than those measured under initial velocity conditions.

We speculate that previous measurements of avian 5'D made under assay conditions that did not reflect initial velocities may have been limiting to different extents with different treatments. Thus, treatment differences could be magnified, decreased or eliminated as a result of assay artifacts. We addressed one example by reinvestigating 5'D activity during development. We found good correspondence between the potential whole liver 5'D per unit body weight and the plasma T_3/T_4 ratios for the stages sampled. Comparisons with previous data indicated limited qualitative similarity in the pattern of 5'D development, however, extreme quantitative differences were present in the activities measured and in the proportional relationships between stages, when unvalidated T_4 assay results were compared with validated rT_3 assay results. These findings suggest the need for reinvestigation of many previous studies of factors affecting avian 5'D.

ACKNOWLDGEMENT

Chapter III

HEPATIC 5′DEIODINASE ACTIVITY OF JAPANESE

QUAIL USING THYROXINE AS SUBSTRATE: ASSAY

VALIDATION.

ARE THERE VALID T4 5′D ASSAYS IN THE LITERATURE?

For any enzyme assay to be valid, enzyme activity measurements must be made under assay conditions (e.g., substrate, cofactor, tissue concentration, and incubation time) in which enzyme activity is proportional to protein (enzyme) concentration and linear with incubation time (see Chapter 2 for a detailed discussion; also Dixon and Webb, 1979; Engel, 1981). Further, proportionality and linearity of enzyme activity must be demonstrated for each treatment group within a study to ensure that valid comparisons are made.

The published studies of T_4 5′D activity can be divided into three categories, as follows: (1) those that include no validation studies, (2) those that include validation studies, but

validated conditions are not used in the routine assay for experimental measurements, and (3) those that include validation studies and use this information in the design of the routine assay. Each category will be discussed below.

Because T_4 is the precursor in the formation of active thyroid hormone, triiodothyronine (T_3), T_4 is the 5'D substrate of physiologic interest. However, I must conclude that the majority of published 5'D assays which use T_4 as substrate are invalid and therefore must be questioned.

(1) No Validation

Many published studies of 5'D do not include validation studies. This is true of early studies of mammalian 5'D that were important in launching the field (1978-1980), as well as much of the comparative literature (which for the most part has adopted methods from these early mammalian studies, without modification). One cannot judge directly the validity of these studies, but strong indications of methodological difficulties are apparent when these are compared with more recent work employing currently accepted techniques.

Characteristically, the assay conditions used in the studies in question consisted of: 25% (w/v) tissue homogenate, final T_4 concentrations of about 0.5 to 1 μM, and incubation times as long as one to two hours (mammals: Harris *et al.*, 1978; Kaplan and Utiger, 1978; El-Zaheri *et al.*, 1980; birds: Decuypere *et al.*, 1983; and Scanes *et al.*, 1983). The detailed studies of Leonard and Rosenberg (1980) described in section (2) below, argue that such conditions do not achieve proportionality between enzyme activity and protein concentration, nor equimolar production of iodide and T_3 from T_4 in mammalian liver and kidney.

(2) Validation Studies Performed but Information Not Used In Experiments

Studies in this category include a reaction time course or proportionality experiment which demonstrates that the reaction state loses linearity with incubation time or lacks proportionality with protein concentration, i.e., that invalid conditions were used in the experimental work. Proportionality experiments are crucial for design and evaluation of

proper enzyme assays, however, these often are omitted from studies of 5'D activity. McNabb *et al* (1986) performed a proportionality experiment using assay conditions typical of those used in early mammalian studies (1977-1980) and in most studies of avian 5'D. They found a lack of proportionality between T_4 5'D activity and homogenate protein concentration in Japanese quail liver. This provides evidence that other studies employing these widely accepted assay conditions, likewise, are invalid.

Leonard and Rosenberg (1978) performed a proportionality study in which they measured T_4 5'D activity in several dilutions of subcellular fractions of rat kidney homogenate. They observed an apparent decrease in enzyme activity with increasing homogenate concentration. They attribute this lack of proportionality to the presence of a soluble inhibitor of the enzyme whose efficacy is reduced upon dilution. Chiraseevenuprapund *et al*. (1978) observed the same behavior in the majority of a series of proportionality experiments employing rat kidney tissues. This latter group demonstrated that the lack of proportionality was not due to the presence of a soluble inhibitor, by dialysis of the tissue preparation that would remove such soluble factors.

Other than these three studies, there are few examples of proportionality experiments. The pattern of enzyme activity observed in these three studies is remarkably similar. Taken together they provide strong evidence to question most studies using T_4.

In contrast to the limited number of proportionality experiments that have been published, it is relatively easy to find examples of studies in which the assay conditions used were limiting (for examples see Rudas, 1986; Eales, 1986; and Chopra, 1977). Each of these studies determined a reaction time course, but the incubation time used in their routine assays was well beyond the period of linearity with time. Thus, enzyme activity reported in these papers reflects limiting conditions that could be due to substrate depletion, product inhibition or enzyme inactivation; i.e., these measurements must be underestimates.

(3) Validation Studies Performed and Information Properly Used

The only published studies of T_4 5'D that demonstrate a proportional relationship between hepatic and renal protein concentration and 5'D activity are those of Wu, *et al.*, (1985, 1986). Wu *et al.* (1985) demonstrated proportionality, using mouse liver tissue, but only for a very limited range of protein concentrations and using only three data points. In similar work using ovine liver tissue, Wu *et al.* (1986) demonstrated an even more limited range of proportionality defined by three data points, consisting of the blanks (no protein) and the two lowest protein concentrations used. In our study of quail hepatic T_4 5'D, we have found similar narrow ranges of apparent proportionality that, upon further study, were shown not to yield proportional relationships.

It is important to note that T_4 can be used as substrate in valid assays in tissues that contain Type II deiodinase activity, e.g. brain, pituitary gland, and brown adipose tissue (BAT). Type II 5'D activity is a true T_4 5'D, i.e. it has highest affinity for T_4, which it 5'-deiodinates, and it lacks 5D capability. Thus, it is easy to achieve a single reaction system with tissues that contain Type II activity, and there are several published studies that demonstrate T_4 5'D proportionality with tissue protein concentration and linearity with incubation time (brain, Leonard *et al.*, 1982, Visser, 1983; pituitary gland, Kaplan, 1980, Koenig *et al.*, 1984, St. Germain *et al.*, 1985; and BAT, Goswami and Rosenberg, 1986, Silva *et al.*, 1987). In contrast, liver and kidney tissue contain Type I 5'D activity which can deiodinate at both the 5- and 5'-positions and thus performs multiple reactions when T_4 is used as substrate.

ATTEMPTED VALIDATION STUDIES OF QUAIL LIVER 5'-DEIODINASE ACTIVITY

In this section I will describe the series of experiments conducted to test the validity of reported methods for measurement of T_4 5'D activity and my subsequent attempts to establish a valid assay. The first series of experiments tested the use of T_3 RIA to measure T_3 produced by deiodination of T_4. When the RIA proved to be inadequate I began a second series of experiments to establish a valid assay using labeled T_4. Finally, I will summarize my conclusions based on these experiments and speculate on the future of the use of T_4 as a substrate for measurement of 5'D activity.

Measurement of T_3 Produced by RIA

A common method for measuring 5'D activity has been to use unlabeled T_4 as substrate and to measure T_3 production by RIA. Many of the studies cited above used T_4 concentrations at, or below, the published K_m for T_4 5'-deiodination. I used this "RIA approach" in my initial experiments, but with T_4 concentrations well above the K_m; thus enzyme activity would approach maximum velocity and enzyme quantity would be the limiting factor evaluated by the assay. I also chose assay conditions so that no more than ten percent of the T_4 would be consumed.

EXPERIMENT 1: Determination of Substrate Concentration that Yields Maximum Velocity. I employed assay conditions typically used in avian T_4 5'D assays (25% liver homogenate and 1 mM dithiothreitol as cofactor) except T_4 concentrations were 2 to 6 μM. The T_3, present in an ethanol extract of the reaction mixture was measured by the ethanol-based RIA in use in our laboratory. Blanks consisted of incubation tubes that were quenched by the addition of ice-cold absolute ethanol immediately following the addition of substrate.

RESULTS: High apparent T_3 concentrations were observed in the incubation blanks. This apparent T_3 background increased with increasing T_4 concentration. Three possible explanations for the high apparent T_3 present in these blanks are: (1) actual T_3 contamination in the substrate preparation, (2) T_4 crossreaction with the T_3 antibody used in the RIA, and (3) T_3 endogenous to the tissue. Triiodothyronine of the highest purity commercially available (Calbiochem-Behring, Inc., La Jolla, CA; $T_3 > 96\%$, $T_4 < 0.1\%$ by HPLC) was purchased, so problems of T_3 contamination seem unlikely. It also seems unlikely that endogenous T_3 contributes significantly, because ethanol extracts from liver tissue, in the absence of exogenous T_4, have T_3 concentrations at or below the limit of sensitivity of our T_3 ethanol RIA (unpublished data, our laboratory). T_4 crossreaction with the T_3 antibody seems the most likely cause of apparent T_3 in the assay blanks. This is addressed by experiment two.

EXPERIMENT 2: Testing for Substances that Crossreact/Interfere with the T_3 RIA. To investigate the possiblity that other substances interfere with the T_3 RIA, I first checked for parallelism between the RIA standard curve and a dilution series of 5'D assay extracts. Lack of parallelism between the two curves would provide strong evidence for the presence of a crossreacting or interfering substance. A dilution series of the highest RIA standard also was analyzed as a check on the analytical technique.

RESULTS: The curve produced by serial dilution of the 5'D assay extract was not parallel with the RIA standard curve; as the tissue extract concentration was reduced it approached the standard curve. The curve prepared from the highest RIA standard was parallel to the standard curve. This indicates that interfering substance(s) were present in the assay extracts. It is likely that T_4 is this interfering substance, because it is present in high concentration. Other native compounds likely to be present in tissues (such as tetraiodothyroacetic acid, triiodothyroacetic acid and triiodopropionic acid), that are known to crossreact with T_3 antibodies, probably would be present in only extremely low concentration (Nejad *et al.*, 1975). Brown *et al.* (1988), when measuring tissue T_3 content, were able to achieve parallelism between a T_3 RIA standard curve and a curve produced by dilution of an tissue extract. However, the endogenous T_4 present in their tissue samples was at much lower

concentration than that in the present study. This is further evidence that T_4 is the interfering substance in my assay when T_4 concentrations are high.

EXPERIMENT 3: T_4 Crossreaction with T_3 Antibody. This experiment was designed to determine whether or not T_4 interferes with the T_3 RIA. Based on a reaction mixture containing 2 μM T_4, the maximum possible T_4 concentration in an ethanol extract of the reaction mixture is 802 ng T_4/ml; the highest T_3 RIA standard is 16 ng/ml. The producer of the T_3 antibody (Endocrine Sciences, Tarzana, CA) used in the RIA claims a T_4 crossreaction of 0.25% (determined by direct incubation of 10 ng T_4 with the T_3 antibody and I^{125}-T_3; results are expressed as amount of T_3 equivalent to 10 ng of T_4). Each standard in a T_3 ethanol standard curve was spiked with T_4 to achieve a T_4 concentration of 800 ng T_4/ml (equivalent to the maximum possible carry over). These T_3 RIA standards, spiked with T_4, were used as samples and were run against the T_3 RIA standard curve.

RESULTS: Greater than 95% displacement of the labeled T_3 from the antibody was observed in all of the T_4-spiked T_3 standards. Thus, crossreaction of T_4 with the T_3 antibody precludes the use of a T_3 ethanol RIA for measuring 5'D activity at high T_4 concentrations. It should be noted that I did not investigate other T_3 antibodies. In the literature there has been very little investigation of RIA performance in this context. In cases where a commercially available T_3 antibody has been used, investigators generally rely on supplier claims of low T_4-crossreactivity. These commercial T_3 antibodies are usually produced for use in clinical serum RIAs, and crossreactivity is stated for a single set of conditions germane to this application.

Measurement of Radioiodine Release from I^{125}-T_4

As an alternative to using unlabeled substrate and T_3 RIA for measuring 5'D activity, others have used substrates labeled with radioiodine and isolated and quantified the products by paper or column chromatography. There have been few attempts to used labeled T_4 in this manner, so experiments were designed to attempt validation studies with this physiologically

relevant substrate. This approach avoids the problem of RIA crossreactivity, can be more sensitive than RIAs, and allows quantification of substrate depletion (and of all labeled products in some separation systems). If equivalency of labeled products (i.e. a single reaction) could be established using systems that isolate all products (e.g. Sephadex column chromatography or paper chromatography), then rapid separations (Dowex columns) that measure only labeled iodide release could be utilized as the final assay.

EXPERIMENT 1: Demonstration of a Single Deiodination Reaction. To determine if T_4 was 5′-deiodinated to only T_3 and iodide, the time course of the deiodination of I^{125}-labeled T_4 was investigated. The labeled products were separated by Sephadex column chromatography and quantified in a gamma counter. Sephadex column chromatography is labor intensive and time consuming; this limits its utility in routine assays. This separation was used to verify the the presence of a single deiodination reaction and to validate the use of anion exchange chromatography (Dowex AG 50W-X8) to isolate radioiodine released from labeled T_4. Anion exchange chromatography is rapid and many samples can be analyzed simultaneously. The quantity of radioiodine isolated by Sephadex column chromatography was compared with that isolated by anion exchange from parallel incubations.

Results: The equimolar production of T_3 and iodide observe in these preliminary experiments suggests that T_4 5′D was the only activity present. If T_4 5-D also were present, it would result in the accumulation of excess iodide, and further deiodination of T_3 would result in a reduction in product T_3 and a further increase in iodide. Iodide accumulation, when measured by anion exchange chromotography, was identical to T_3 accumulation. When separated by Sephadex column chromatography there appeared to be slightly more iodide than T_3 at each time sampled. However, it has been reported that several related compounds coelute with, or very near to, iodide during Sephadex column chromatography under the conditions used here (Smallridge, *et al.*, 1981). If small amounts of these compounds were present, the "apparent" iodide peak would overestimate the actual iodide content of the sample. Results of these experiments suggested that an assay to measure labeled iodide

production from labeled T_4 was feasible. The next set of experiments attempted to validate such an assay.

EXPERIMENT 2: Proportionality of T_4 5′D Activity with Hepatic Protein Concentration: Fixed Incubation Times. Several different preparations of liver tissue [crude homogenate; supernatant following homogenate centrifugation at 1000g (hereafter referred to as 1000g supernatant); postmitochondrial fraction (supernatant after homogenate centrifugation at 12,100g); microsomes] were diluted to give a wide range of protein concentrations. These preparations were incubated with cofactor and labeled T_4 (as substrate) for a fixed incubation period.

RESULTS: In no case were we able to demonstrate a proportional relationship between protein concentration and T_4 5′D activity. The rate of product accumulation decreased with increasing protein concentration and reached a plateau, usually between 4 and 8% substrate consumption. The activity of 5′D was not observed to be proportional to protein cocnetration for any range of protein concentrations or for any of the different tissue preparations tested. To verify that this lack of proportionality was not caused by substrate or cofactor limitation, this experiment was repeated using the 1000g supernatant at several different T_4 concentrations (up to 18 μM), or at several different cofactor concentrations (up to 20 mM DTT). In no case was proportionality observed.

EXPERIMENT 3: Proportionality of T_4 5′D Activity with Hepatic Protein Concentration: Variable Incubation Times. This experiment was identical to experiment 2, except only 1000g supernatant was used. The 1000g supernatant was incubated with 4μM T_4 and 1 mM DTT; incubation time was varied inversely with protein concentration. The rationale for this experiment was that similar proportions of substrate degradation could be achieved by this strategy, and overcome what might be apparent assay limitations and lack of proportionality.

RESULTS: In several runs of this experiment, we were able to produce a proportional relationship by adjusting the incubation time for each protein concentration. While this procedure produces a proportionality between T_4 5′D activity and protein concentration, it does not represent useful strategies for analyzing data. With experimental samples, one

would need to know the 5'D activity and the relationship between 5'D activity and protein concentration to determine the proper incubation time for each individual sample.

EXPERIMENT 4: Time Course of T_4 5'D Activity. This experiment was designed to determine if product accumulation was linear with incubation time and, if so, to clearly define the limit of this range under the assay conditions employed. Various concentrations of 1000g supernatant were incubated with 4 μM T_4 and 1 mM DTT and replicate incubations were stopped at regular intervals.

RESULTS: In all cases, we observed an initial period of linearity between T_4 5'D activity and incubation time. However, the activity reached a plateau between 4 and 10% substrate consumption. As in previous experiments, T_4 5'D activity was not proportional to protein (enzyme) concentration; e.g., the difference between activities measured at different supernatant concentrations was not proportional to the difference in supernatant concentration.

EXPERIMENT 5: Inhibition of T_4 5'D Activity by T3. To determine if product inhibition caused the rapid loss of linearity and lack of proportionality, 1000g supernatant was incubated with 4 μM T_4 and 1 mM DTT in the presence of increasing concentrations of T_3 (up to 4 μM which would represent 100% substrate consumption).

RESULTS: No inhibition of T_4 5'D activity was observed in the presence of up to 4 μM added T_3. Therefore, product inhibition was not the cause of the lack of proportionality and the relatively low substrate consumption observed (always <10%).
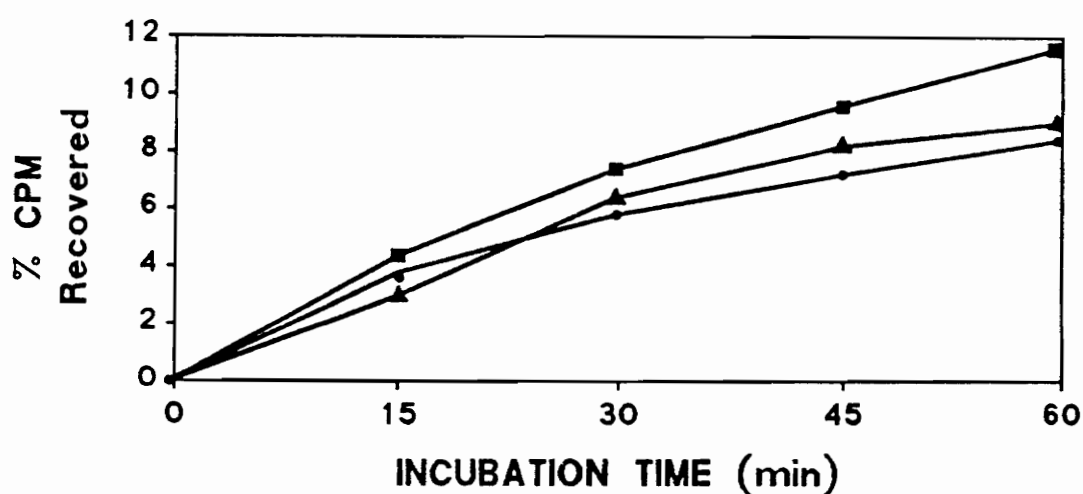
Figure 5. Time course of hepatic T_4 5'D activity: Liver was pooled from five adult Japanese quail. Iodide (-■-) and T_3 (-●-) were isolated by Sephadex column chromatography; Iodide (-▲-) in duplicate, parallel incubation tubes was isolated by Dowex column chromatography. Assay conditions: 4 μM T_4, 1 mM dithiothreitol, 18 mg tissue/ml in the reaction mixture. Data reported are individual determinations (Sephadex) or means of triplicate determinations (Dowex). Data are expressed as percent cpm recovered.
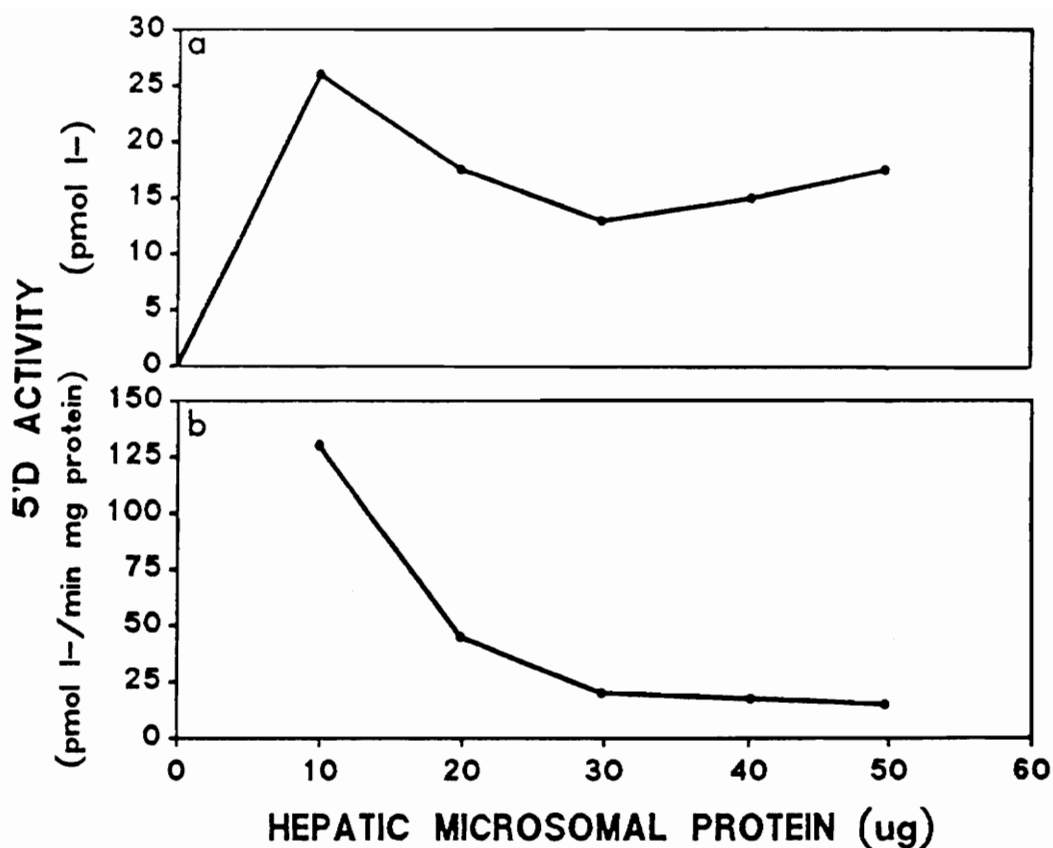
ASSAY VALIDATION WITH THYROXINE AS SUBSTRATE

Figure 6.   Attempt to demonstrate proportionality of hepatic T₄ 5'D activity with enzyme (microsomal protein) concentration: Liver was pooled from five adult Japanese quail. Assay conditions: 4 μM T₄, 1 mM dithiothreitol, 30 min incubation. Data reported are the mean of triplicate determinations. (a) pmol iodide generated in 30 min, (b) pmol iodide generated per min-mg protein.

SUMMARY

I conclude that T_4 cannot be used as substrate for studying 5'D activity in liver and kidney tissue using any of the methods described here. Hgh concentrations of unlabeled T_4 remaining in reaction mixture extracts preclude the use of RIAs for measuring T_3 produced during the 5'D assay.

An assay using radiolabeled T_4 as substrate was evaluated. This method allows accurate quantification of radioiodide release, and preliminary studies suggested that T_4 was degraded only to T_3 and labeled iodide. However, continued studies revealed a lack of proportionality between the presumed T_4 5'D activity and tissue protein (enzyme) concentration.

There are several possible causes of this lack of proportionality when T_4 is used as substrate. The two simplest causes could be product inhibiton or substrate limitation. However, we have demonstrated that product inhibition by T_3 does not occur under the conditions used. The presence of endogenous inhibitors of 5'D activity within the tissue also seems unlikely. Most such inhibitors would be found in the soluble fraction of a tissue homogenate, so microsomal preparations should not contain such factors. Our proportionality experiments using microsomes yielded the same results as other homogenate preparations, and argue against the existence of inhibitors causing the lack of proportionality observed. Substrate limitation seems equally unlikely, given that $>90\%$ of the substrate remains at the end of incubation. Similarly, in experiments where T_4 concentrations were increased to as high as 36 μM, increases in percent substrate consumption were not observed. Similar experiments demonstrate that cofactor also is not limiting.

These studies argue that, in quail liver, as in mammalian kidney and liver (experiments of Leonard and Rosenberg, 1980), when T_4 is used as substrate in 5'D assays multiple deiodination reactions occur (5D and 5'D), and are the reason why proportionality cannot be established. It is possible that the balance between the predominance of the 5'D and 5D

pathways changes under different conditions. The results of our experiments analyzed by Sephadex column chromatography suggest that only a single reaction occurs; however, only a very limited range of assay conditions was tested. Specifically, the ratio of iodide to T_3 produced was not measured at different protein concentrations.

The problems raised by these studies have received little previous attention in the literature. Despite the attempts by Leonard and Rosenberg (1980), the problems with using T_4 as substrate have not been adequately reviewed in the published literature, and many investigators continue to use invalid T_4 5'D assays (see review by McNabb and Freeman, 1989). Currently, the only solution to these difficulties has been the use of rT_3 as an alternative substrate in 5'D assays using liver and kidney tissue. The simple deiodination pathway of rT_3 makes it relatively easy to establish valid assay conditions when used as substrate. rT_3 assays have been widely adopted for studies of mammalian liver and kidney, but much comparative work has continued to utilize unvalidated T_4 assays.

Future investigation of T_4 5'D will require the use of methods such as HPLC, which can separate T_4 and rT_3, as well as other thyroid hormone metabolites. The ability to measure the accumulation and futher deiodination of the products of T_4 deiodination within a single reaction system would provide a valid estimate of T_4 5'D activity. Comparison of estimates of T_4 and rT_3 5'D activity in the same samples would test the hypothesis that rT_3 5'D activity is a valid reflection of T_4 5'D activity. This assumption is taken for fact;this has never been verified.

Further, tracing the cascade of deiodinase reactions within a single reaction would give a much clearer picture and deeper understanding of the pathways of TH deiodination and their relative importance. To date we have been limited to examining pieces of the entire cascade isolated from the whole. We could test the model constructed from these data and very likely extend the model. Changes in the pathways of deiodination due to physiological and environmental factors can be characterized in fine detail. It would allow investigation of regulation at the cellular level by manipulating the availablity of substate(s), singly and in combination, as well as the concentration of putative cofactors and other effectors.

HPLC separation and fluorescence detection could be used to measure tissue TH concentration. This would require pre-column derivatization of TH in the samples with dansyl cholride to make them fluoresce. This would allow measurement of substrate availability and investigation of the relationship between tissue 5′D activity and 5′D activity. This would test the hypotheses concerning the role of tissue 5′D in the regulation of tissue T_3 availability in various tissues.

HPLC methods, though powerful, are costly to set-up and labor intensive, therfore, the questions must be asked thoughtfully and evaluated to give the greatest advancement of our understanding. Our laboratory is currently working to develop methods for measurement of TH by HPLC.

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Professional Employment

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Honors and Awards

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Financial Support

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1985-1986, Sigma Xi Grant-in-aid of Research - Peripheral T_3

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Membership in Professional Organizations

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Activities in Professional Organizations

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Comparative Endocrinology, American Society of Zoologists

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Presentations at Scientific Meetings

Freeman, T.B. and F.M.A. McNabb. 1986. Preliminary
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in quail. Va. J. Sci. **37**: 64. Presented at Vir.
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Freeman, T.B. 1986. Triiodothyronine (T_3) production by liver
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Research Symposium, Virginia Polytechnic Institute and
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Freeman, T.B., L.C. Stallard, M.J. McNichols and F.M.A. McNabb.
1987. Evaluation of methimazole (MMI)-induced hypothyroidism
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Freeman, T.B., L.C. Stallard, M.J. McNichols and F.M.A. McNabb.
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hypothyroidism in Japanese quail. Presented at the national
meeting of American Society of Zoologists, New Orleans, LA,
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propylthiouracil (PTU) sensitivity of the iodothyronine
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Eastern Regional Conference on Comparative Endocrinology,
Lancaster, PA, May, 1988.

Stock, M.K., J. Metcalf, T.B. Freeman and F.M.A. McNabb. 1989.

Plasma thyroid hormone concentrations in growth-altered

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McNichols, M.J., T.B. Freeman, J.D. Rieman and F.M.A. McNabb. 1989.

Hepatic 5′deiodinase activity in Japanese quail and ring doves:

Characterization studies with T_4 and rT_3 as

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May 14-20, 1989.

Freeman, T.B., J.D. Rieman and F.M.A.McNabb. 1990. Relationship

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Presented at the national meeting of the American Society of

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McNabb, F.M.A., T.B. Freeman, E.A. Dunnington, P.B.Siegel and S.

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Research Papers

McNabb, F.M.A., E.A. Dunnington, T.B. Freeman and P.B. Siegel. 1990.

Thyroid hormones and growth patterns of embryonic and posthatch

chickens from lines selected for high and low juvenile body

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McNabb, F.M.A. and T.B. Freeman. 1990. Comparative studies of thyroid hormone deiodinase systems. In: "Progress in Comparative Endocrinology" (A. Epple, C.G. Scanes and M.H. Stetson, eds.) **342**: 433-438.

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